The Finnish Red Cross Blood Service
and Faculty of Biological and Environmental Sciences,
Doctoral Programme in Integrative Life Science
University of Helsinki
Finland

CULTURE SYSTEMS AND QUALITY PARAMETERS FOR CLINICAL-GRADE MESENCHYMAL STROMAL CELLS

Sofia Oja

ACADEMIC DISSERTATION

To be presented for public examination with the permission of
the Faculty of Biological and Environmental Sciences of
the University of Helsinki,
in Auditorium 2 of the Haartman Institute
Haartmaninkatu 3, 00290 Helsinki
on October 9th 2020 at 12 noon.

Helsinki 2020

ACADEMIC DISSERTATIONS FROM THE FINNISH RED CROSS BLOOD SERVICE, NUMBER 66

Supervisors:
Adjunct professor Matti Korhonen, MD, Ph.D.
Finnish Stem Cell Registry
Finnish Red Cross Blood Service
Helsinki, Finland

Adjunct professor Johanna Nystedt, Ph.D.
Advanced Cell Therapy Centre
Finnish Red Cross Blood Service
Helsinki, Finland

Follow-up group:
Professor Susanna Miettinen, Ph.D.
Faculty of Medicine and Health Technology
University of Tampere
Tampere, Finland

Professor Elina Ikonen, MD, Ph.D.
Department of Anatomy
University of Helsinki
Helsinki, Finland

Reviewers:
Adjunct professor Susanna Narkilahti, Ph.D.
Faculty of Medicine and Health Technology
University of Tampere
Tampere, Finland

Guido Moll, Ph.D.
Berlin-Brandenburg Center for Regenerative Therapies
Charité Universitätsmedizin
Berlin, Germany

Opponent:
Professor Heli Skottman, Ph.D.
Faculty of Medicine and Health Technology
University of Tampere
Tampere, Finland

Custos:
Professor Kari Keinänen, Ph.D.
University of Helsinki
Helsinki, Finland

ISBN 978-952-5457-52-0 (print)
ISBN 978-952-5457-53-7 (PDF)
ISSN 1236-0341
http://ethesis.helsinki.fi
Helsinki 2020
Unigrafia

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Castrén E., Sillat T., **Oja S.**, Noro A., Laitinen A., Konttinen Y.T., Lehenkari P., Hukkanen M., Korhonen M. Osteogenic differentiation of mesenchymal stromal cells in two-dimensional and three-dimensional cultures without animal serum. Stem Cell Research and Therapy 2015; 6:167.

II Laitinen A., **Oja S**., Kilpinen L., Kaartinen T., Möller J., Laitinen S., Korhonen M., Nystedt J. A Robust and reproducible animal serum-free culture method for clinical-grade bone marrow-derived mesenchymal stromal cells. Cytotechnology 2016 Aug;68(4):891-906.

III **Oja S.**, Komulainen P., Penttilä A., Nystedt J., Korhonen M. Automated Image Analysis Detects Aging in Clinical-grade Mesenchymal Stromal Cell Cultures. Stem Cell Research and Therapy 2018 Jan 10;9(1):6.

IV **Oja S.**, Kaartinen T., Ahti M., Korhonen M., Laitinen A., Nystedt J. The utilization of freezing steps in mesenchymal stromal cell (MSC) manufacturing: potential impact on quality and cell functionality. Frontiers in Immunology 2019 July; 10:1627.

The publications are referred to in the text by their Roman numerals.

ABBREVIATIONS

AT	Adipose tissue
ALP	Alkaline phosphatase
ATMP	Advanced Therapy Medicinal Product
BM	Bone marrow
CDK	Cyclin-dependent kinase
CFU-F	Colony-forming unit-fibroblast
CFSE	Carboxyfluorescein succinimidyl ester
CO_2	Carbon dioxide
CPA	Cryoprotective agent
2D	2-dimensional
3D	3-dimensional
DMSO	Dimethylsulfoxide
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal calf serum
FBS	Fetal bovine serum
FRCBS	Finnish Red Cross Blood Service
GMP	Good manufacturing practices
GvHD	Graft-versus-Host disease
HSA	Human serum albumin
IBMIR	Instant blood-mediated inflammatory reaction
IDO	Indoleamine 2 3-dioxygenase
IFN-γ	Interferon-gamma
IL	Interleukin
ISCT	International Society for Cellular Therapies
kbp	Kilobase pair (1000 base pairs)
kDa	Kilodalton
MNC	Mononuclear cell
MSC	Mesenchymal stromal (stem) cell
OCN	Osteocalcin
OD	Optical density
OIS	Oncogene-induced senescence
$p16^{INK4a}$	Cyclin-dependent kinase inhibitor 2A
$p21^{Cip1/Waf1}$	Cyclin-dependent kinase inhibitor 1 (CDK-interacting protein 1)
PD	Population doubling
PFA	Paraformaldehyde
PL1	Platelet lysate 1
PL2	Platelet lysate 2
PLP	Platelet lysate and plasma; see PL2
PRP	Platelet-rich plasma; see PL1
RS	Replicative senescence

Runx2	Runt-related transcription factor 2
SA-β-gal	Senescence-associated beta-galactosidase
SA-DNAm	Senescence-associated DNA methylation
SAHF	Senescence-associated heterochromatin foci
SASP	Senescence-associated secretory phenotype
SIS	Stress-induced senescence
TF	Tissue factor
TRF	Terminal restriction fragment
UCB	Umbilical cord blood
UC	Umbilical cord
WJ	Wharton's jelly

ABSTRACT

During the two last decades, mesenchymal stromal cells (MSCs) have been topics of intensive investigation due to their intriguing characteristics, which make them exciting prospects for cell-based therapies. MSCs possess immunomodulatory functions and a broad differentiation capability, meaning that they have several potential therapeutic applications, such as a systemic treatment for immunological disorders or locally administered to correct tissue defects.

The most widely studied and utilized characteristic of MSCs has been their immunomodulatory property. Graft-versus-Host disease (GvHD), a severe and highly lethal complication following allogeneic stem cell transplantation, has been one of the first indications where the use of MSCs has been widely explored. MSCs have also been evaluated for the treatment of Crohn's disease, multiple sclerosis, and chronic inflammation. The repair of bone and cartilage defects is also another application of significant interest.

The cell therapy field has taken tremendous steps from research laboratories towards the clinic, hand in hand with the development of a whole new branch of the biotechnology industry. Regulations for GMP manufacturing of advanced therapy medicinal products (ATMPs) have been framed in conjunction with the development of MSC therapies and the evaluation of the risks associated with the manufacturing process have been complemented with accumulated knowledge. According to the regulatory authorities, a risk- and evidence-based approach should be used to evaluate safety risks and sources of alterations or impairments in product functionality.

Culturing MSCs *ex vivo* is relatively simple; however, culturing conditions and handling of the cells can modify their characteristics and functionality. This fact sets limitations on the manufacturing and demands a deep understanding of the mechanisms affecting cell products. The selection of suitable cell sources, culture medium supplements, and optimization of culturing conditions are the key parameters to be considered if one wishes to achieve high yields of functional MSCs. During the last fifteen years, the safety of MSC products has been improved by replacing fetal bovine serum (FBS) as a medium supplement with human origin platelet lysates (PLs); in this way one can obtain MSCs which do not express bovine xenoantigens and do not pose a risk of transferring animal pathogens.

As is the case with all somatic cells, also MSCs age during the expansion process. Natural aging, called replicative senescence, is known to limit the lifespan of MSCs and to reduce their functional properties. However,

senescence may also be triggered by external sources such as DNA damaging agents, oxidative stress, or sub-optimal culturing conditions. Because the progression of aging impairs the cellular functions and alters the secretory profile of the cells, the presence of senescent cells in MSC cultures is undesirable. Therefore, careful monitoring of cellular aging during MSC expansion is essential for product quality.

If one wishes to ensure efficient distribution and appropriate-timed dosing of MSC products, manufacturing of MSCs as "off-the-shelf" products will be necessary but requires cryopreservation of the cells. Cryopreservation of the intermediate or the final product allows a predictable manufacturing scheme and improves the availability of the released products. However, the effect of cryopreservation on the functionality and aging of the MSCs is a topic of intensive debate and requires more investigation.

This study has focused on determining optimal expansion conditions for clinical-grade bone marrow (BM) MSCs by using platelet-derived culture medium supplements. We also explored the potential of PL to promote osteogenic differentiation of MSCs as compared to FBS supplemented culture. We also aimed to improve product quality by introducing an imaging-based screening method to detect aging-related morphological changes from the MSC cultures. Finally, we investigated how any additional freezing steps during the manufacturing process of BM-MSCs would affect the basic manufacturing parameters and alter the cellular aging process.

We found that PL prepared of pooled fresh platelets and plasma with two freeze-thaw cycles effectively supported MSC expansion and maintained their functionality in an ambient oxygen concentration. We also observed that PL could promote osteogenic differentiation at least equally with FBS in 2D cultures and slightly better in a 3D culture system. We were able to detect and quantify aging-related morphological changes from MSCs cultures and demonstrated that the rapid increase in cell size evident after passage 5 was indicative of the expression of primary senescence markers. When the effect of interim freezing steps was investigated, we found that interim freezing at passages 0 and 1 did not alter the basic manufacturing parameters and did not accelerate the initiation of senescence. Our results suggest that the manufacturing process for clinical-grade MSCs may be scaled up by using additional freezing steps in the early passages.

This study was a part of the manufacturing development process during the establishment of The Advanced Cell Therapy Centre in the Finnish Red Cross Blood Service and has yielded insights into the establishment and scaling up of PL supplemented culture system for clinical-grade MSCs. In addition, our screening method for senescent cells could be implemented into clinical-grade

manufacturing of MSCs and be a useful tool as an in-process monitoring method for cell quality.

1 REVIEW OF THE LITERATURE

1.1 MESENCHYMAL STROMAL CELLS

Mesenchymal stromal cells (MSCs) are multipotent cells, which reside in the bone marrow and the stroma of multiple organs and tissues. They are a heterogeneous population of cells, which comprise a small proportion of self-renewing stem cells and stromal cells in various differentiation stages. Upon plating on standard tissue culture plastic, the MSCs adhere to the plastic surface and form colonies of cells with a fibroblast-like morphology. MSCs possess a differentiation potential to at least mesodermal lineage cells as well as a capability to modulate immune functions by producing a large variety of secreted and membrane-bound factors (Dominici et al. 2006; Uccelli et al. 2008; Viswanathan et al. 2019).

The first indication of MSCs was presented in the 18th century by the German pathologist Julius Fredrich Cohneim, who characterized a non-hematopoietic, adherent, and spindle-shaped cell population within hematopoietic cells (Cohneim 1867). Soon after Cohneim's observations, in 1869, Emile Goujon showed that these BM-derived cells had an intrinsic osteogenic potential (Goujon 1869). The first clear evidence of MSCs was provided by Alexander Friedenstein in 1976 when he analyzed precursor cells from BM and showed that these fibroblast-like cells formed clonal colonies and could be cultured for several passages. Friedenstein called these cells osteogenic precursor cells since they could differentiate into osteoblasts. Furthermore, these cells could also differentiate into adipocytes and chondrocytes. (Friedenstein 1976).

Despite the relatively long history of MSCs, many of their *in vivo* functions are still unclear, and also the definition of MSCs has been questioned (Sacchetti et al. 2016; da Silva Meirelles et al. 2008; Elahi et al. 2016; Reinisch et al. 2015). MSCs reside in all postnatal tissues, but whether all of these cells are MSCs remains unclear (da Silva Meirelles et al. 2006; Sacchetti et al. 2007; Bernal and Arranz 2018; Kozlowska et al. 2019). The term MSC was initially restricted to BM-derived cells, but nowadays the abbreviation has been widened to describe cells also from many other sources such as from adipose tissue (AT), umbilical cord blood (UCB) and umbilical cord (UC), placenta, skin or dental pulp (Viswanathan et al. 2019).

MSCs are not restricted to humans and have been well characterized also in mice (Baddoo et al. 2003), rats (Santa María et al. 2004), baboons (Devine et al. 2001), pigs (Moscoso et al. 2005), cows (Bosnakovski et al. 2005) and horses (Ringe et al. 2003). The therapeutic potential of MSCs has been tested, especially in large mammals (Dazzi et al. 2006).

1.1.1 ORIGIN OF MSCS

Currently, there are two distinct descriptions of the origin and properties of MSCs. The first model describes MSCs as progenitor cells that are found in the BM. These progenitors can form in a hematopoietic microenvironment and differentiate into skeletal tissues (Friedenstein 1976; Bianco et al. 2013; Sacchetti et al. 2007). The second model was suggested by Arnold Caplan and described MSCs as progenitors for multiple tissues, also tissues other than skeletal types (Caplan 1991). Caplan also introduced the name "mesenchymal stem cell" to underline the self-renewal property of MSCs. However, only a minor population of MSCs are capable of self-renewal, and therefore, the name mesenchymal stromal cell or multipotent mesenchymal stromal cell has been chosen as being more appropriate (Dominici et al. 2006; Horwitz et al. 2005).

According to the conventional description of MSCs, they are multipotent progenitor cells originating from the mesoderm of the embryonal inner cell mass and have a restricted potential to differentiate into lineage-committed cells (Figure 1.). Recent reports suggest that instead of one universal MSC, cells from different tissue sources have a distinct transcriptome and functionality, although they share the essential criteria of MSCs (Sacchetti et al. 2016; Reinisch et al. 2015; Grégoire et al. 2019; Jin et al. 2013; De Almeida et al. 2016; Elahi et al. 2016). MSCs from different tissue origins have also been reported to vary according to their phenotype, proliferation potential, expression of cell surface markers, differentiation capacities, and secretome (Ribeiro et al. 2013; Kozlowska et al. 2019; Elahi et al. 2016; Moll et al. 2019; Banfi et al. 2000; Pittenger et al. 1999b).

Non-BM-MSCs have been suggested to originate from pericytes and to express similar surface antigens to MSCs isolated from skeletal muscles, placenta, AT, and pancreas (Crisan et al. 2008; Caplan 2008). These pericytes could also differentiate into osteoblasts, adipocytes, chondrocytes, and muscle cells (Crisan et al. 2008). A subpopulation of MSCs express CD146, which is a marker for pericytes (Mo et al. 2016).

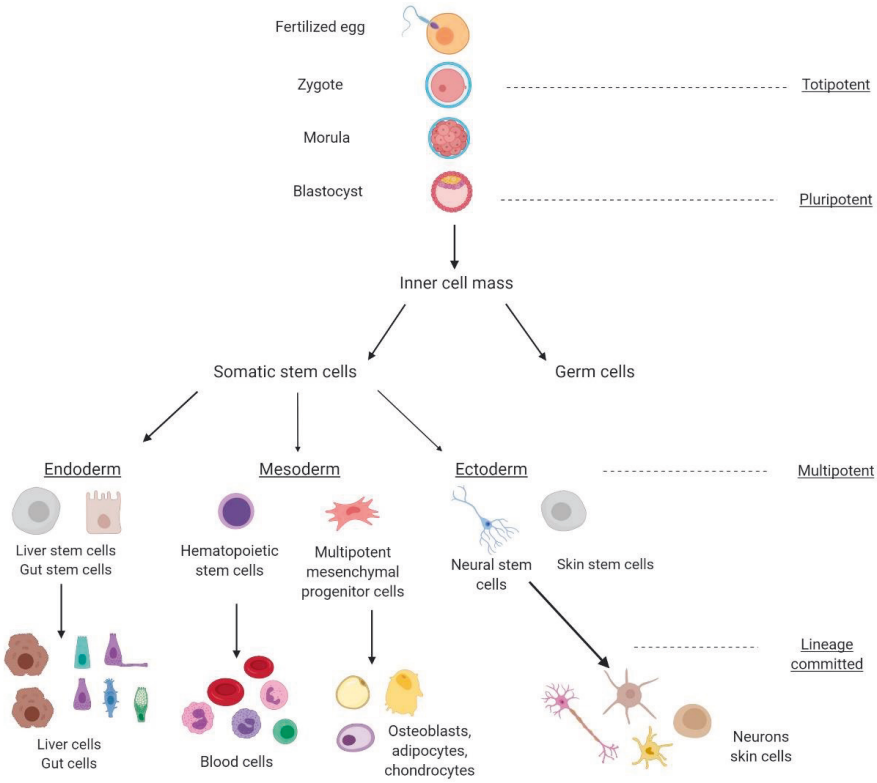

Figure 1. Hierarchy of stem cells. Multipotent mesenchymal progenitor cells (MMPC) originate from the inner cell mass of a blastocyst. Both germ cells and somatic stem cells, such as hematopoietic stem cells (HSCs), originate from the mesodermal layer of the inner cell mass. MMPCs and HSCs, are multipotent stem cells indicating that they can self-renew, and can differentiate into various lineage-specific cell types. The differentiation potential of multipotent stem cells is restricted to one or more specialized cell types. The figure is modified from (Hayes et al. 2012; Tyndall and Uccelli 2009).

1.1.2 MSC SOURCES

MSCs are usually isolated from BM, but there they comprise only 0.001-0.01% of the BM mononuclear cells. Cells with MSC-like characteristics have been later isolated from the stroma of multiple tissues and organs, such as from AT (Zuk et al. 2002), UC (Sarugaser et al. 2005) or UCB (Erices et al. 2000), placenta (In ’t Anker et al. 2004), skin (Shih et al. 2005), the dental pulp (Gronthos et al. 2000), synovial membrane (De Bari et al. 2001), breast milk (Patki et al. 2010), and amniotic fluid (In ’t Anker et al. 2003).

The best-characterized MSCs are the BM-derived MSCs. However, the method for collecting BM aspirate is invasive, and therefore also other sources

for MSCs used in clinical applications have been considered. The most widely studied alternative sources are AT and other birth-related tissues such as UC, UCB, and placenta (Kern et al. 2006; Troyer and Weiss 2008; Parolini et al. 2008). MSCs are rare in UCB, but for example, placenta, either decidua or other placental compartments, or UC, provide a rich and ethically sustainable source of stromal cells and have therefore been a source of great interest (Bieback and Brinkmann 2010; Parolini et al. 2008; Mattar and Bieback 2015).

Several studies comparing the isolation and properties of BM-, AT-, UCB- and UC-MSCs have been published (Bieback et al. 2008; Wagner et al. 2005; Jin et al. 2013; Elahi et al. 2016; Kern et al. 2006; Heo et al. 2016; Reinisch et al. 2015; Sacchetti et al. 2016; Grégoire et al. 2019). These studies reported differences in *in vivo* frequencies, proliferative potentials, and functional properties. UCB-MSCs display a tremendous expansion capacity over BM- and AT-MSCs, but the isolation of these cells and the establishment of the cultures is challenging (Bieback et al. 2008; Jin et al. 2013). The MSC frequency in the UCB is the lowest; the success rate in the establishment of the cultures has varied between 20-60%, whereas BM- and AT-MSCs cultures have been mostly established with a 100% success rate (Bieback et al. 2008). UCB-MSCs can be cultured for more extended periods than BM- or AT-MSCs. AT-MSCs show, however, shorter doubling times at each passage, when compared to BM- and UCB-MSCs (Jin et al. 2013). MSCs from all the three sources express senescence markers, but UCB-MSCs with much lower levels than BM- or AT-MSCs (Jin et al. 2013).

Although MSCs from different sources may express similar morphologies and surface antigen composition, they vary according to their differentiation capacity, as well as gene expression and epigenetic profiles (Bieback and Netsch 2016; Reinisch et al. 2015; Sacchetti et al. 2016). The study of Reinisch et al. showed the BM-derived MSCs have distinct expression and methylation patterns from those of AT-, UC- or skin-derived MSCs. BM-MSCs were also the only MSCs, which could form an ectopic HSC microenvironment when compared to MSC from AT, UC, or other sources such as from skin or dental pulp (Reinisch et al. 2015). According to that analysis, BM-MSCs are more closely related to AT-MSCs than to UC- or skin MSCs (Reinisch et al. 2015). In addition, a study by Sacchetti et al. revealed distinct gene expression profiles of UCB-, periosteum-, muscle- and BM-derived MSCs (Sacchetti et al. 2016).

A comparison of the immunomodulatory properties of BM, AT, UCB, and WJ originating MSCs is difficult because of the different experimental settings used in the experiments. Many studies have reported the more robust immunomodulatory properties AT-MSC than BM-MSCs. MSCs derived from fetal origins (UC, UCB, and placenta) have been shown to modulate immunological functions more efficiently than AT-MSCs (Bieback and Brinkmann 2010; Jin et al. 2013; Mattar and Bieback 2015). BM-MSCs seem to have a lower immunomodulatory capacity than that of UCB-MSCs or AT-MSCs.

However, they can produce enough immunosuppression for clinical use as shown in early-stage clinical trials (Le Blanc et al. 2004; Ringdén et al. 2006; Le Blanc et al. 2008).

The most suitable tissue origin of MSCs should be selected when MSCs are considered for therapeutic applications because tissue-specific characteristics may have a marked impact on the efficacy or safety of MSCs (Moll et al. 2019, 2020b). For example, the expression of tissue factor TF/CD142 varies depending on the cell source. TF/CD142 is the main reason for the incompatibility of MSCs with blood and it triggers blood clotting and can also induce an Instant blood-mediated inflammatory reaction (IBMIR). The expression of TF/CD146 is low in BM-MSCs, and much higher in AT-, UC- and in placenta-derived MSCs (Moll et al. 2020b, 2019; Grégoire et al. 2019), and therefore BM-MSC may be the most suitable choice for systemic infusions.

1.1.2.1 *The hematopoietic stem cell niche*

Stem cells usually reside in a "nest" called the niche, which forms a protective environment for them. The niche maintains stemness by keeping the cells in a non-differentiated state and supports their physiological functions. The niche-forming cells regulate the survival, self-renewal, migration, and differentiation of the stem cells via various mechanisms such as through growth factors, chemokines, and extracellular matrix molecules and cell contacts (Uccelli et al. 2008). The main components of the HSC niche are pericytes, myofibroblasts, BM stromal cells, osteocytes, osteoblasts, and endothelial cells (Muguruma et al. 2006).

Different subtypes of stromal cells can be found in the BM. Sachetti et al. identified $CD146^+$ cells in the sinusoidal wall area with a colony-forming unit (CFU-F) activity and an ability to transfer the BM microenvironment when transplanted into mice (Sacchetti et al. 2007). Cells expressing CD271 can be found in the trabecular regions of the BM. These cells can also form colonies, transfer the BM microenvironment, and can differentiate into mature mesodermal cell types (Sacchetti et al. 2007; Tormin et al. 2011).

HSC niche has been demonstrated to consist of two types of compartments with different functions located at distinct areas in the BM. The endosteal niche is lined by osteoblasts in low oxygen areas of BM, near the bone's surface. The endosteal niche contains $CD271^+$ and $CD271^+/CD147^{low/-}$ stromal cell subsets, and HSCs, which are maintained long-term in a quiescence state (Tormin et al. 2011). The vascular niche is located around the sinusoidal area and is enriched with $CD146^+$ and $CD271^+/CD146^+$ stromal cells together with activated and fast proliferating hematopoietic stem progenitor cells (HSPCs)(Tormin et al. 2011). The proportion of different stromal cell subtypes changes upon aging;

$CD271^+/CD146^{low/-}$ subset is found in adults, subtype $CD271^+/CD146^+$ is expressed in cells from children and fetuses, and the third subtype, $CD271^-/CD146^+$, can be detected only from fetal BM (Maijenburg et al. 2012).

The HSC niche controls the differentiation and proliferation of stem cells (Crippa and Bernardo 2018). MSCs produce high levels of HSC maintaining cytokines such as stromal cell-derived factor 1 (CXCL12; C-X-C motif chemokine-12), stem cell factor (SCF or Kit ligand), angiopoietin-1, IL-7, and vascular cell adhesion molecule-1 (VCAM-1) and osteopontin (Arai et al. 2004;). When new blood cells are needed, HSCs will mature into progenitor cells in the vascular niche and be released into the circulation through the sinusoids (Uccelli et al. 2008) (Figure 2.).

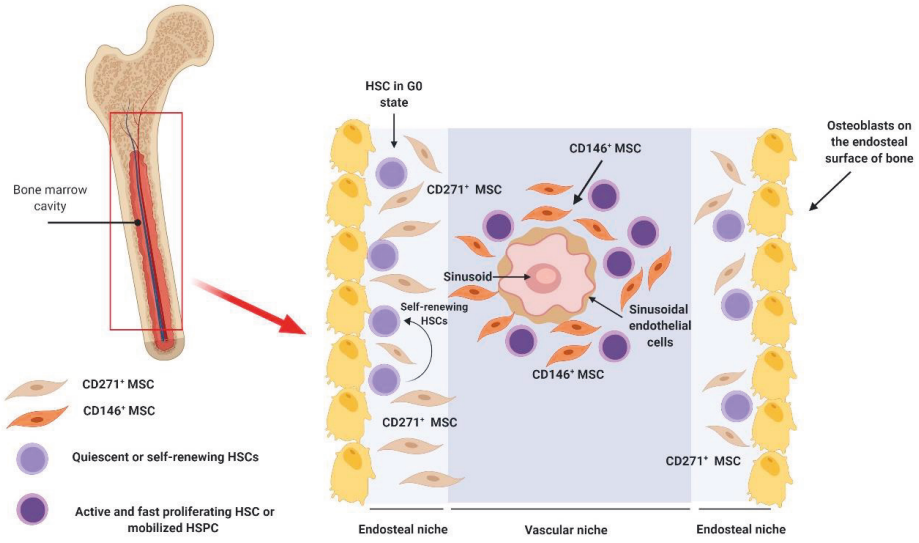

Figure 2. A simplified description of the subtypes of stromal cells in the HSC niche. BM comprises two distinct areas with different functions. $CD271^+$ and $CD271^+/CD146^{low/-}$ MSCs maintain HSCs in the non-proliferative G0 state in hypoxic conditions (light blue areas) until hematopoietic progenitor cells are needed. CD146+ and $CD271^+/CD146^+$ MSCs are enriched in vascularized regions of BM and regulate the mobilization of hematopoietic progenitors to the circulation through the sinusoids. The figure is modified from (Sacchetti et al. 2007; Tormin et al. 2011).

1.1.3 CRITERIA FOR MSCS

MSCs have been found in almost all tissues, and due to the lack of specific markers for MSCs, isolation, and identification of these cells may be difficult. MSCs are known to express a large variety of surface markers, which however are not unique for MSCs, but are shared by other cell types (Mo et al. 2016). Due to the heterogeneity of MSC populations, the International Society for Cell and Gene Therapies (ISCT) has defined the minimum criteria for MSCs. The first

definition was published in 2006, but upon the improvement of the methodology and thus increasing knowledge, the guidelines for defining MSCs have been updated (Dominici et al. 2006; Viswanathan et al. 2019). The minimal criteria have been set for *in vitro* cultured MSCs to facilitate uniform characterization of MSCs and most likely do not reflect the *in vivo* properties of MSCs (Mo et al. 2016).

According to the current definition, MSCs are plastic adherent fibroblastoid cells with a spindle-shaped morphology. *In vitro* cultured MSCs should express surface antigens CD105, CD73, and CD90 and lack the expression markers such as CD34, CD45, CD14 or CD11b, CD79α or CD19 to exclude the presence of contaminating cell subsets, such as epithelial cells, as well as cells from lymphoid, myeloid and endothelial lineages (Dominici et al. 2006; Viswanathan et al. 2019) (Figure 3.). According to the ISCT criteria, MSCs should differentiate into osteoblasts, adipocytes, and chondrocytes *in vitro* (Pittenger et al. 1999a; Dominici et al. 2006).

In addition to the *in vitro* markers, CD271 has also been used as a marker for primitive MSCs. However, CD271 can be applied for freshly isolated MSCs before *in vitro* culturing, because its expression is lost upon culturing and when MSCs are exposed to atmospheric oxygen (Álvarez-Viejo et al. 2015; Elahi et al. 2016). BM-MSCs that reside in the vascular niche of the BM express CD146 (Sacchetti et al. 2007). CD146 is also shared by pericytes, which share many characteristics with MSCs. It has been debated whether MSCs are pericytes or if pericytes are the *in vivo* progenitors of MSCs (Crisan et al. 2008; Caplan 2017, 2008).

According to the ISCT criteria, MSCs should not express HLA-DR; however, HLA-DR expression can be detected if MSCs are cultured with human platelet-derived supplements (Grau-Vorster et al. 2019; Laitinen et al. 2016a) (Figure 3.). Cell surface HLA-DR expression is a plastic condition, and it is also elicited if MSCs are exposed to IFN-γ, which is found in the inflammatory environment (Polchert et al. 2008; Stagg et al. 2006). Furthermore, the lack of expression of CD34 is not imperative, since it can be detected depending on donors and culture passages, but can also be detected from an early culture of AT-derived MSCs (Viswanathan et al. 2019) (Figure 3.).

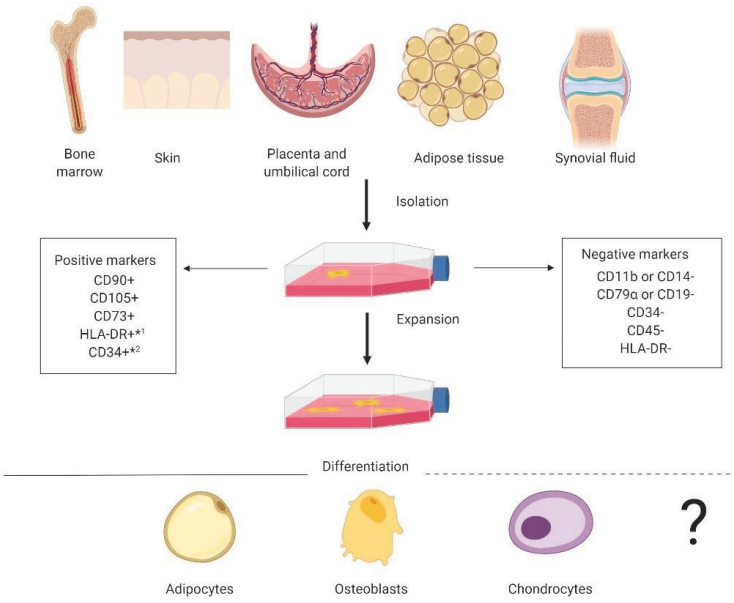

Figure 3. Minimal criteria for defining MSCs. MSCs can be isolated from various sources, and in *in vitro* culture, they are plastic adherent fibroblastoid cells lacking expression of lymphoid, myeloid, endothelial, and epithelial markers. MSCs possess differentiation potential to adipocytes, osteoblasts, and chondrocytes (Dominici et al. 2006). However, depending on the culture system and used cell source, MSCs may express HLA-DR antigen (*1) and CD34 (*2) deviating from the ISCT criteria published in 2006 (Grau-Vorster et al. 2019; Laitinen et al. 2016b; Viswanathan et al. 2019). Whether MSCs have wider differentiation potentials remains unclear (Sacchetti et al. 2016).

According to recent reports, cells that share surface antigen composition characteristic for MSCs may vary according to their epigenetic profiles and differentiation properties (Sacchetti et al. 2016; Reinisch et al. 2015). The definition and nomenclature of MSCs have been updated and clarified, along with accumulated knowledge since the term mesenchymal stem cells was introduced in 1991 (Caplan 1991) (Figure 4.). To acknowledge this more accurate characterization, ISCT has continuously revised the MSC criteria and recommends denoting the tissue origin together with the abbreviation “MSC” and to clarify whether the abbreviation is used for stem or stromal cells. Due to the variability of MSC populations, it is evident that one specific marker for MSCs cannot be found, and therefore the identity of MSCs is confirmed by a set of identity tests (Dominici et al. 2006; Viswanathan et al. 2019). The identity of MSCs should be verified using a comprehensive assay matrix, which includes a quantitative RNA analysis of selected genes, flow cytometric surface antigen analysis, as well as proteome analysis and epigenetic profiling ((Viswanathan et al. 2019).

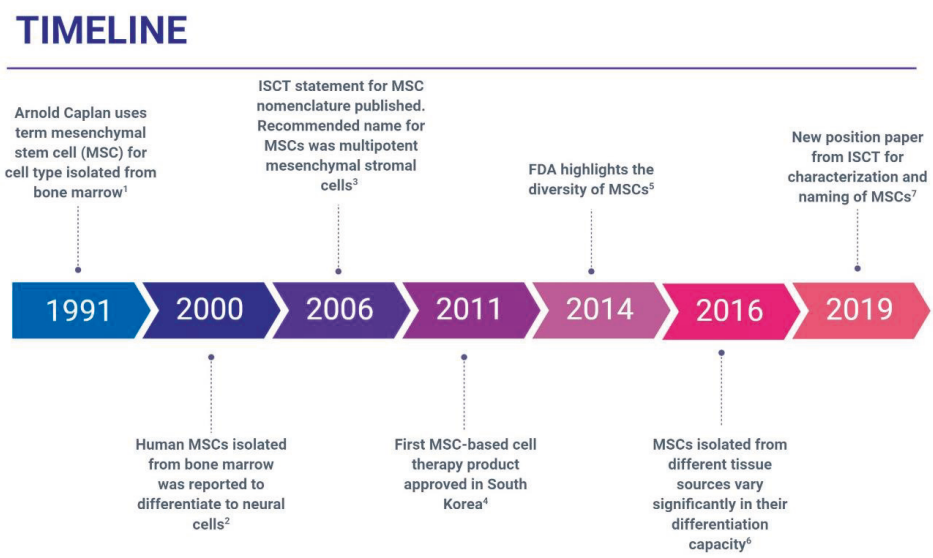

Figure 4. Timeline for defining MSCs. The figure is partly adopted from (Sipp et al. 2018). References for timeline steps; [1](Caplan 1991), [2] (Woodbury et al. 2000), [3](Dominici et al. 2006), [4](Yang 2011), [5](Mendicino et al. 2014), [6](Sacchetti et al. 2016), [7](Viswanathan et al. 2019).

1.1.4 DIFFERENTIATION POTENTIAL

Arnold Caplan suggested in 1991 that MSCs could theoretically differentiate into a large variety of tissues; bone, cartilage, tendon, ligament, adipocytes, dermis, muscle, BM stroma, and connective tissue (Caplan 1991). The differentiation of adult MSCs into mesodermal lineage cells, such as osteoblasts, chondrocytes, and adipocytes, has been repeatedly confirmed by *in vitro* assays (Jaiswal et al. 1997; Purpura 2004; Johnstone et al. 1998; Pittenger et al. 1999). However, differences in the efficacy of differentiation due to tissue origin have been reported. BM-MSCs have been shown to differentiate robustly into all three lineages in various studies (Bieback et al. 2008; Wagner et al. 2005; Jin et al. 2013; Heo et al. 2016), whereas UC- and UCB-MSC have been reported to differentiate only to osteogenic and chondrogenic directions, but not into adipocytes (Bieback et al. 2008; Kern et al. 2006). AT-MSC differentiate preferably to adipocytes and, to a lesser extent, to osteoblasts and chondrocytes (Zajdel et al. 2017).

Many studies have reported a broad differentiation potential, especially for BM- and AT-MSCs, and demonstrated that under certain conditions, MSCs could also differentiate into tissues other than those of mesodermal origin. MSCs were reported to differentiate into skeletal muscle (Dezawa et al. 2005), tendon (Kuo and Tuan 2008), myocardium (Shim et al. 2004), smooth muscle (Jeon et al. 2006), and endothelial cells (Oswald et al. 2004). Some studies have

also shown that MSCs can, in certain conditions, differentiate into neurons (Krampera et al. 2007) or epithelial cells (Timper et al. 2006). However, many of the studies showing differentiation potentials to cells other than mesodermal lineages have been performed in *in vitro* cultures with selected inducing agents, which favor specific differentiation directions, and many of these studies have not been confirmed by *in vivo* results (Strioga et al. 2012; Quinn and Flake 2008). In addition, even though MSCs might have expressed tissue-specific markers, the differentiated cells have not been able to express the characteristics and functionality of mature cells (Phinney and Prockop 2007; Volz et al. 2016; Fitzsimmons et al. 2018; Krampera et al. 2007). Therefore, the differentiation shown in *in vitro* experimental settings does not necessarily reflect the true differentiation potential of MSCs.

The conception of MSCs as a uniform source of progenitors of multiple tissues has been, however, challenged by recent studies (Reinisch et al. 2015; Sacchetti et al. 2016). Newer studies have used *in vivo* transplantation experiments, and transcriptome analyses to show the impact of tissue origin on the MSC functionality, despite fulfilling the minimal criteria listed by ISCT. Studies by Sacchetti et al. and Reinisch et al. characterized an inherent property of BM-MSCs to generate bone and BM stroma (Sacchetti et al. 2016; Reinisch et al. 2015). BM-MSCs were also shown to differentiate into adipocytes, which may support the HSC microenvironment *in vivo* (Reinisch et al. 2015). According to Sacchetti et al., BM-MSCs do not form myocytes and are not spontaneously chondrogenic (Sacchetti et al. 2016). Reinisch et al., however, reported that when BM-MSCs were compared AT-, UC-MSC, only BM-MSCs exhibited a chondrogenic differentiation transcriptional program (Reinisch et al. 2015). Sacchetti et al. also reported that UC-MSCs could form cartilage and bone inherently and that MSCs isolated from muscles were inherently myogenic but were not skeletogenic (Sacchetti et al. 2016).

Despite the large variety of studies demonstrating the differentiation potentials of MSCs, the true *in vivo* nature of MSCs is unclear. MSCs' homing efficacy is low, but some of the intravenously injected MSCs have been found from the injury site (Karp and Leng Teo 2009; Strioga et al. 2012). Animal studies have also shown that MSCs mediate tissue reconstitution without differentiation or fusion to the target organ (reviewed in Strioga et al. 2012). Therefore, it is likely that when MSCs are transplanted to the injury site, they can promote tissue regeneration by paracrine activity rather than through differentiation.

1.1.4.1 *Markers for osteogenic differentiation of MSCs*

Ex vivo expanded MSCs have been evaluated for treating bone defects. Osteogenic differentiation can be induced by exposing culture-expanded MSCs to β-glycerophosphate, dexamethasone, and ascorbic acid 2-phosphate (Jaiswal et al. 1997). A variety of transcription factors regulate osteogenic differentiation, such as Runx2, which is the primary controller of differentiation, but also osterix and β-catenin (Almalki and Agrawal 2016). The osteogenic differentiation of MSCs can be divided into three phases. The characteristic for the first phase is the increase in cell numbers. In the second phase, an increase in the expression of alkaline phosphatase (ALP) is observed. ALP is required for the formation of the collagen matrix. The final phase of the differentiation results in a high expression of osteocalcin (OCN) and osteopontin and is followed by calcium and phosphate deposition into the collagen matrix (Birmingham et al. 2012).

1.1.5 CELLULAR CROSSTALK BETWEEN MSCS AND THE IMMUNE SYSTEM

MSCs secrete a large variety of soluble and membrane-bound factors, some of which exert immunomodulatory functions. MSCs can affect and modulate both innate immunity and adaptive immunity, and in general, MSCs have an immunosuppressive effect (Uccelli et al. 2008; Le Blanc and Mougiakakos 2012; Singer and Caplan 2011). However, after certain stimuli, they may also act as proinflammatory cells (Stagg et al. 2006). The multifaceted interactions of MSCs with immune cells are summarized in Figure 5.

T cells interact with DCs and other antigen presenting cells to activate the T-cell receptor and co-stimulatory receptor signaling, which leads to a rapid proliferation of T cells and the production of effector cell-activating cytokines (Chiesa et al. 2011). CD8+ mediated cytotoxicity is activated because of costimulation. Effector CD4+ cells produce the Th1 and Th17 cell subsets, which enhance the inflammatory signal (Ghannam et al. 2010).

Innate immunity acts as a first-line barrier against pathogens and factors jeopardizing tissue integrity. Natural killer cells (NK cells) are innate immunity cells, which produce proinflammatory cytokines and are cytotoxic to infected and cancer cells (Glenn 2014). The properties of NK cells are intricately regulated by a balance between activating and inhibitory receptors, e.g. they are activated by exposure to IL-2 and IL-15 originating from the action of inflammatory cytokines IFN-γ and TNF-α on T cells (Spaggiari et al. 2006). MSCs can directly block IL-2 and IL-15 induced proliferation of NK cells through indoleamine 2,3-deoxygenase (IDO) and prostaglandin E2 (PGE2), MSCs can also block cytokine production and the expression of activating receptors in NK cells, but cannot, however, inhibit the cytotoxicity of freshly isolated NK cells (Moretta et al. 2001; Sotiropoulou et al. 2006; Spaggiari et al. 2006).

BM-derived MSCs promote the movement of monocytes and macrophages from BM to the sites of injury (Chen et al. 2008). Monocytes entering an inflammatory site will respond to inductive signals by polarizing to either the M1 (pro-inflammatory) or the M2 macrophage subsets (anti-inflammatory). M1 macrophages secrete IFN-γ and TNF-α; these are agents that support and enhance the inflammation signals, whereas M2 macrophages promote the switch to the anti-inflammatory and regenerative state by secreting IL-10 and TGF-β1 (Le Blanc and Davies 2015). MSCs can promote polarization either through inducing a shift from the M1 to the M2 subset or directly from monocytes (Gao et al. 2014). MSC-induced direct maturation is initiated by cell-cell contacts but also by secreted factors such as PGE2, IL-6, and IDO, (Karp and Leng Teo 2009; Maggini et al. 2010; Németh et al. 2009). MSCs can induce the downregulation of the costimulatory molecules CD86 and MHC II and increase the secretion of IL-10 and IL-6 in the polarized M2 macrophages (François et al. 2012b; Kim and Hematti 2009; Maggini et al. 2010). IL-10 secretion prevents neutrophil migration to the inflammatory site and thus reduces oxidative stress. M2 polarization also induces the production of regulatory T cells (Treg) (Melief et al. 2013). Recent studies have indicated that MSCs can influence the Treg proliferation through cell-cell contacts with T cells and APCs, as well as through the secretion of soluble factors and exosomes (Negi and Griffin 2020).

Dendritic cells (DC) connect the innate and adaptive immunity by secreting cytokines and serving as APCs, of which DCs are the primary type. After phagocytosing pathogens, DCs mature, become activated, and upregulate the expression of MHC and costimulatory molecules. Activated DCs present the antigens to T cells, but also directly activate NK cells and B cells (Dubois et al. 1999; Gerosa et al. 2002). MSCs can affect DCs, both directly and indirectly. MSCs prevent the maturation of monocytes and CD34+ precursor cells to DCs and reduce the receptors needed for DC maturation, such as MHC II and CD80 and CD86 (Chiesa et al. 2011; Du Rocher et al. 2012). As a result of exposure to MSCs, DCs are unable to activate T-cells due to the reduced ability to present antigens, to stimulate T-cell proliferation, and to induce maturation of naïve T-cells (Beyth et al. 2005; Du Rocher et al. 2012). MSCs can also return mature DCs to an immature form, where antigen presenting and co-stimulatory molecules are downregulated, IL-12 is secreted, and DCs are unable to stimulate the proliferation of lymphocytes (Zhang et al. 2009).

MSCs can inhibit the T cell proliferation independently of the MHC molecules by arresting them at the G0/G1 cell cycle phase (Benvenuto et al. 2007; Glennie et al. 2005). MSCs do not induce apoptosis in T cells but can promote the survival of overstimulated T cells, which are led to activation-induced cell death (Benvenuto et al. 2007). Inhibition of T cells has been shown to reduce the secretion of IFN-γ both *in vivo* and *in vitro* (Aggarwal and Pittenger 2005) and

to increase the production of anti-inflammatory IL-4 by T_H2 cells (Aggarwal and Pittenger 2005; Zappia et al. 2005). Therefore, MSC shift the balance from pro-inflammatory T cell activities and cytotoxicity towards anti-inflammatory functions. Anti-inflammatory T cells functions are also driven by stimulating the production of regulatory CD4+CD25+ T cells (Tregs). MSCs can induce the generation of Tregs either indirectly, by inducing IL-10 production by plasmacytoid DCs, which then stimulate the generation of Tregs (Aggarwal and Pittenger 2005; Fadeel et al. 1998), or directly by the secretion of HLA-G5 antigen (Selmani et al. 2008).

Controversial results have been published from experiments exploring the interactions between B cells and MSCs. The reason for the variable results has been hypothesized to be due to variable experimental settings in the *in vitro* studies (Augello et al. 2005; Corcione et al. 2006; Glennie et al. 2005; Rasmusson et al. 2007). However, most studies indicate that MSCs can inhibit B cell proliferation as well as their differentiation to antibody-producing plasma cells and expression of cytokine receptors (Augello et al. 2005; Corcione et al. 2006; Glennie et al. 2005). The inhibitory effects seem to be due to the release of soluble factors and cell-cell contacts between MSCs and B cells (Corcione et al. 2006). Other investigators have reported that MSCs could support survival, proliferation, and differentiation of B cells in healthy individuals and in children with systemic lupus erythematosus (Rasmusson et al. 2007; Traggiai et al. 2008).

In addition to secreted factors and cell-cell contacts, MSCs can exert their immunomodulatory function through exosomes and a wide range of extracellular vesicles (EVs), which can transport a large variety of mRNA and non-coding RNA (Weiss et al. 2019). The functionality of the exosomes is dependent on their cargo. Exosomes can activate or suppress the immune system by affecting cytokine secretion, differentiation, and polarization of immune cells or activation of T cells (Weiss et al. 2019; Moll et al. 2020b).

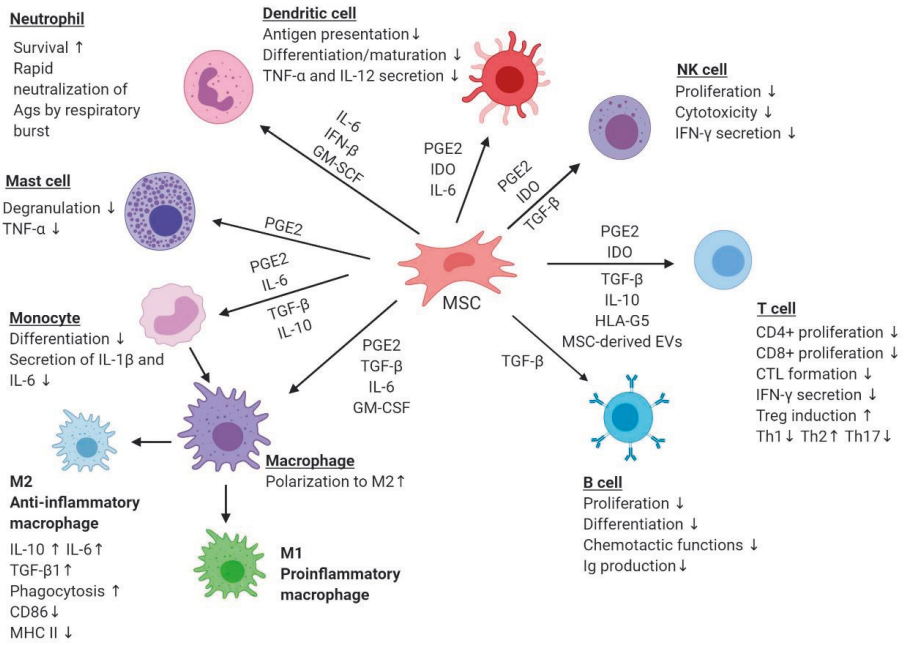

Figure 5. Overview of the immunomodulatory effects of MSCs on innate and adaptive immunity. The figure summarizes the publications referred to in paragraph 1.1.6.

1.1.6 MOLECULAR MECHANISMS UNDERLYING MSC-INDUCED IMMUNOSUPPRESSION

MSCs secrete various soluble factors, either constitutively or when stimulated by target cells. IFN-γ, TNF-α, and IL-1β secreted by the T cells have been identified as significant initiators of the immunomodulatory activities of MSCs (Krampera et al. 2006; Németh et al. 2009; Singer and Caplan 2011; Redondo-Castro et al. 2017). MSCs constitutively secrete Transforming growth factor β (TGF-β), hepatocyte growth factor (HGF), IL-10, PGE2, IL-6, IL-8, and HLA-G5, which affect both directly and indirectly to proliferation, differentiation, and function of innate and adaptive immunity cells (Figure 5.) (Di Nicola et al. 2002; English et al. 2007; Selmani et al. 2008; Aggarwal and Pittenger 2005; Németh et al. 2009).

Resting MSCs express low levels of MHC I molecules and lack the expression of MHC II molecules and costimulatory molecules CD40, CD80, and CD86 entirely (Krampera et al. 2003; Prasanna et al. 2010). Exposure of MSCs to low concentrations of IFN-γ upregulates the expression of MHC II and may convert MSCs into antigen-presenting cells (Stagg et al. 2006). However, although the

expression of MHC II molecules MSCs does not express CD80 or CD86, which are essential costimulatory molecules (Stagg et al. 2006; Galipeau 2017).

MSCs exert most of their immunosuppressive effects by producing IDO, but some of the activity may be mediated by IL-6 (Meisel et al. 2004; Moll et al. 2014a). IDO catalyzes the cleavage of tryptophan to kynurenine. Because tryptophan is essential for cell survival, proliferation, and protein synthesis, especially for lymphocytes, cleavage of tryptophan impairs the proliferation of T cell subsets, NK cells, and dendritic cells (Meisel et al. 2004). *In vitro*, MSCs immunosuppressive activities can be activated (primed/licensed) by IFN-γ treatment (François et al. 2012a).

Moll et al. described in 2012 that when MSCs are administered intravenously, they become recognized by the complement system and destroyed due to IBMIR (Moll et al. 2012). In 2014 Moll et al. also hypothesized that dying MSCs may be useful in triggering phagocytosis due to their ability to recognize apoptotic and complement opsonized cells and turn them into the polarized M2 type regulatory cells (Moll et al. 2014a). Galleu et al. showed later that those GvHD patients, who had the highest T cell-mediated cytotoxic reactions towards MSCs, had the best responses to the MSC therapy and suggested that GvHD patients could be treated with apoptotic MSCs (Galleu et al. 2017). However, the effect of apoptotic MSCs can only be seen if the cells had been infused intravenously and but not by intraperitoneal delivery, as in the study by Galleu et al. (Galipeau and Sensebé 2018). The model of the immunosuppression mediated by fit and apoptotic MSCs is illustrated in Figure 6.

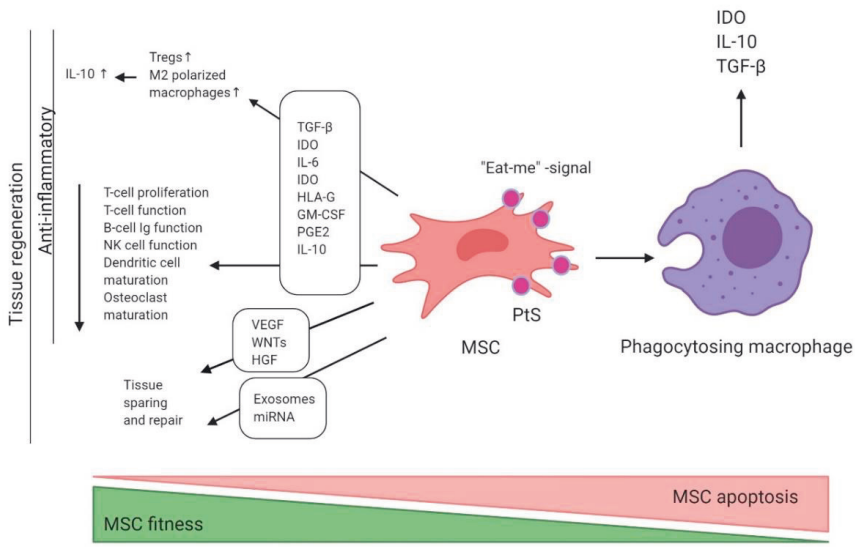

Figure 6. Suggested model for MSC immunosuppression mediated by fit and apoptotic cells. According to the conventional immunosuppression model, culture adapted and fit MSCs exert anti-inflammatory functions through secreted factors, which result in an increase in the numbers of Tregs and M2 macrophages as well as a reduction in immune cell proliferation and cytotoxic activities. Together with these functions, MSCs participate in tissue regeneration by expressing morphogens and exosomes. According to the model suggested by Galleu et al. intraperitoneally administered apoptotic MSCs present "eat-me" signals produced by phosphatidylserine (PtS) to the phagocytosing macrophages, which in turn secrete anti-inflammatory factors. Figure modified from (Galipeau and Sensebé 2018) and harmonized with Figure 5.

1.1.7 UTILIZATION OF MESENCHYMAL STROMAL CELLS IN CLINICAL APPLICATIONS

1.1.7.1 *MSCs for GvHD*

One of the prime indications on which the clinical translation of MSCs has first focused because of their immunomodulatory properties is acute and chronic GvHD. Lazarus and coworkers were the first to test MSCs as medicines to treat GvHD in 1995 when culture expanded autologous MSCs were administered to a patient. However, this study was an early clinical phase I study and therefore, did not aim to study clinical efficacy but rather to investigate the safety and feasibility of the MSC therapy (Lazarus et al. 2005). Le Blanc et al. took significant steps in clinical utilization of MSCs in 2004 when a 9-year old boy with severe grade IV aGvHD was treated with allogeneic MSCs obtained from a third-party donor. Treatment with MSCs showed striking results with excellent immunosuppressive effect and rapid healing of gut symptoms (Le Blanc et al.

2004). Subsequently, Le Blanc et al. reported good outcomes in a phase II trial where 55 patients with steroid-resistant acute or chronic GvHD were treated with allogeneic BM-MSCs received from a sibling or third-party donor (Le Blanc et al. 2008).

Subsequent studies have, however, shown heterogeneous results, and evidence for the clinical benefit of MSCs has not been totally convincing (Fisher et al. 2019; Chen et al. 2015; Tolar et al. 2010). Nonetheless, the comparison of pre-clinical and early clinical studies with late clinical studies is challenging due to different manufacturing scales and handling procedures (Galipeau and Sensebé 2018). Meta-analyses evaluating the safety aspects of treatment with MSCs have indicated, however, that they are well-tolerated, and severe adverse effects have not been reported (Lalu et al. 2012; Chen et al. 2015; Fisher et al. 2019).

The long journey of MSCs from bench to the bedside through three industry-sponsored clinical trials has been recently reviewed by Galipeau (Galipeau 2020). The first major industry-sponsored phase III clinical trial was performed between 2006 and 2009. In the study, cryopreserved allogeneic BM-derived MSCs (remestemcel-L, Prochymal®) were provided by Orisis Therapeutics Ltd. (Columbia, MD, USA) for treating adults and children with steroid-refractory GvHD (NCT00366145). The results of the study were initially presented in a conference abstract (Martin et al. 2010) and finally reported much later (Kebriaei et al. 2019). The study did not, however, meet the primary endpoint of a durable complete response lasting at least 28 days, and the difference between responders (30%) and placebo group (35%) was statistically non-significant ($p=0.42$) (Kebriaei et al. 2019). However, a subset study from the data by Kurtzberg et al. revealed that children were responsive to MSCs (Kurtzberg et al. 2010).

The results of the second major study were reported by Kurtzberg et al. The study was initiated in 2007 and enrolled until 2015. The study was distinct from the first Prochymal® study. Children and adults with any grade B and D GvHD were treated if they showed symptoms of steroid resistance for at least three days (NCT00759018). The treatment resulted in an overall response of 65.1% (complete responders 14.1%, partial responders 51.3%) on day 28, and day 100 survival could be predicted from the day 28 results (Kurtzberg et al. 2014).

In 2013 Osiris Therapeutics sold the assets of Prochymal® to Mesoblast Ltd. (Melbourne, Australia). A new open-label, single-arm phase III study (NCT02336230) was initiated with MSCs (remestemcel-L) with a new product name Ryoncil®. The study involved pediatric patients with acute steroid refractory GvHD and was completed in 2017. The study successfully met the primary endpoint of an improved day 28 overall response rate of 69% with patients with severe disease. A significant improvement ($p=0.0003$) was found

compared to the control group, and the effect was sustained at day 180. The results of the study have been reported recently (Kurtzberg et al. 2020).

In 2012, based on results reported by Kurzberg et al. (Kurtzberg et al. 2010), the MSC product received conditional market approval in Canada for children with GvHD, and later in New Zealand and Japan (Galipeau and Sensebé 2018). After obtaining the results from the latest phase III study (Kurtzberg et al. 2020) Mesoblast Ltd. has filed a Biologics License Application for Ryoncil® to the FDA for marketing authorization in USA, Europe, and Asia excluding Japan, to treat children with steroid-refractory acute GvHD (Galipeau 2020). The latest encouraging results show that MSC products may finally meet the FDA requirements for marketing authorization.

1.1.7.2 *MSCs for other indications*

Because MSCs are one of the crucial contributors to the HSC niche, they have been utilized in supporting HSC engraftment after transplantation. MSCs can enhance engraftment by reconstituting the damaged microenvironment. They can also prevent graft rejection by suppressing T cell activity (Ball et al. 2007). The first clinical studies for MSCs as a supporter of HSC engraftment and graft survival revealed a rapid hematopoietic recovery (Koç et al. 2000; Lazarus et al. 2005). Similar results have also been reported in other phase I/II clinical studies (Ball et al. 2007; MacMillan et al. 2009). The study of Bernardo et al. did not, however, detect improved recovery in patients who had received allogeneic BM-MSCs or UCB-MSCs after HSC transplantation compared to the control group (Bernardo et al. 2011). Furthermore, later studies reported heterogeneous and controversial results regarding the ability of MSCs to support engraftment, although the use of MSCs was found to be both feasible and safe. A meta-analysis by Kallekleiv et al. concluded that no statistically significant benefit of MSCs in co-transplantation with hematopoietic stem cells was evident (Kallekleiv et al. 2016).

The secreted anti-inflammatory cytokines, as well as the ability of MSCs to home to injured tissue and to stimulate the recovery of the tissues are the properties which make MSCs promising for cell-based therapy for IBD. The generally accepted cause for IBD is the immune response to environmental factors in genetically susceptible individuals. The therapeutic effects of MSCs in IBD have been studied first in animal models, where they have shown promising results (Algeri et al. 2015). Although controversial results for the efficacy of MSCs have been reported in early clinical trials, an industrial-sponsored phase III clinical trial, where Crohn's disease-related fistulas were treated with MSCs, showed significant efficacy as compared to placebo (Panés et al. 2016; Algeri et al. 2015). The effect was still evident after one year (Panés et al. 2018). A meta-analysis of studies investigating MSCs for treating Crohn's disease-related

fistulas have shown improved healing rates compared to patients who did not receive MSCs and also indicated the safety of MSC therapy (Cao et al. 2017; Panés et al. 2016).

The osteogenic differentiation potential has been utilized in tissue regeneration, especially in treating large bone defects as well as oral and maxillofacial defects (Gimbel et al. 2007; Kaigler et al. 2013; Mesimäki et al. 2009). MSCs have the potential to increase osteoinduction and osteogenesis and have also been used together with PRP to provide healing promoting factors to the injury site in the bone (Yamada et al. 2004). MSCs have been administered systemically, and by injecting locally, but the most promising approach has been the combinatorial therapy with MSCs and a biodegradable scaffold (Oryan et al. 2017). Currently, due to the lack of published clinical trials, controversies in the results, and variability in the methods, protocols, and treatment strategies, MSCs are not yet a viable option in bone regeneration (Oryan et al. 2017).

The use of MSC therapy has also been explored in liver diseases, especially for liver fibrosis (Alfaifi et al. 2018), to improve heart function after myocardial infarction (Lalu et al. 2018) or after ischemic heart failure (Michler 2018; Kastrup et al. 2017). The utilization of immunomodulatory properties has been studied in the treatment of multiple sclerosis (Scolding et al. 2017), as well as in neurodegenerative diseases, where also the regenerative potential of MSC is being evaluated (Volkman and Offen 2017). Most recently, MSCs have shown potential for treating symptoms of a severe acute respiratory syndrome caused by Coronavirus-2 (SARS-CoV-2), such as acute respiratory distress syndrome, pneumonia and sepsis (Moll et al. 2020a).

1.1.7.3 *Translational challenges*

A variety of factors such as different culturing conditions, medium supplements, tissue origin of MSCs, handling and cryopreservation of cells as well as the route of administration may have an impact on the clinical outcomes (Moll et al. 2020b). The source of the tissue is known to have a significant impact on the expression of TF/CD146 on the cell surface. Highly procoagulant Tissue factor is known to be the primary cause of blood incompatibility of MSCs, causing the IBMIR (Moll et al. 2019). Suboptimal culturing conditions and extensive expansion of MSCs may accelerate cellular aging and thus reduce MSCs functionality. The reasons for the modest results of the main study have been widely hypothesized, and one of the major causes has been suggested to be the extensive expansion of MSCs and the use of cryopreserved instead of fresh cells (Galipeau and Sensebé 2018; Galipeau 2013).

MSCs for the first Prochymal® study were expanded extensively to obtain 10 000 doses from one donor, whereas early-stage studies have been based on approximately ten doses per donor (Galipeau and Sensebé 2018). Therefore the final product used in the Prochymal® study may have contained a larger proportion of senescent cells than in the small scale studies (Galipeau and Sensebé 2018). Prochymal® was also a cryobanked product, which was thawed and administered at the point of care. Cryopreservation of MSCs is known to impair the immunomodulatory properties of MSCs (François et al. 2012a). It could be argued that MSC therapies could be improved not only by careful optimization of manufacturing protocols and monitoring of cell products during processing, but also by developing accurate measures for potency, clinical outcomes. Examples of clinical studies utilizing MSCs have been listed in Table 1.

The study presented in this thesis has been linked to the development of the manufacturing process for clinical-grade MSCs, which are to be used for treating children and adults with acute or chronic GvHD in Finnish university hospitals. This study has also concentrated on gathering more knowledge about crucial quality attributes such as cellular aging and the effects of freezing during the production process of MSCs. The treatment provided by the products manufactured in FRCBS Advanced Cell Therapy Centre has been approved by the competent national authority, Fimea, under the hospital exemption license. The results from the follow-up for treating GvHD with allogeneic BM-MSCs have been reported in Salmenniemi et al. (Salmenniemi et al. 2017). The following chapters describe important parameters and quality attributes in the manufacturing of clinical-grade MSC products and finally report the results of our study.

Table 1. Examples of clinical studies performed using MSCs.

Indication	MSCs	Reference
GvHD	Allogeneic BM-MSCs form HLA identical or haploidentical sibling or third-party HLA mismatched donors.	Le Blanc et al. 2004 Ringdén et al. 2006 Le Blanc et al. 2008 von Bonin et al. 2009 Martin et al. 2010*[1] Kurtzberg et al. 2010*[1] Lucchini et al. 2010 Kurtzberg et al. 2014*[2] Dotoli et al. 2017 Salmenniemi et al. 2017 Kurtzberg et al. 2020*[3]
Crohn's disease	Autologous and allogeneic AT- and BM-MSCs	Garcia-Olmo et al. 2009 Duijvestein et al. 2010 Forbes et al. 2014 Dhere et al. 2016 Panés et al. 2016*[4] Panés et al. 2018*[5]
Multiple sclerosis	Autologous BM-MSCs	Karussis et al. 2010 Yamout et al. 2010 Bonab et al. 2012 Connick 2012
Systemic lupus erythematosus (SLE)	Allogeneic BM-MSCs Allogeneic UCB-MSCs	Liang et al. 2010 Wang et al. 2014
Type I diabetes	Autologous BM-MSCs Allogeneic UCB-MSCs	Carlsson et al. 2015 Cai et al. 2016
Osteoarthritis	Autologous BM-MSCs	Orozco et al. 2013 Soler et al. 2016 Al-Najar et al. 2017
Myocardial infarction/Ischemic heart failure	Allogeneic AT-MSCs Autologous and allogeneic BM-MSCs	Hare et al. 2012 Bartunek et al. 2013 Chullikana et al. 2015 Kastrup et al. 2017
Liver diseases	Allogeneic BM-MSCs	Kharaziha et al. 2009 Detry et al. 2017
Kidney transplantation	Allogeneic AT-MSCs Autologous and allogeneic BM-MSCs	Perico et al. 2011 Vanikar et al. 2014 Reinders et al. 2015
Engraftment support after HSCT	Allogeneic BM-MSCs	Koç et al. 2000 Ball et al. 2007 MacMillan et al. 2009 Bernardo et al. 2011 Batorov et al. 2015 Castello et al. 2018

*[1] An industry-sponsored clinical trial with Prochymal® for adult and pediatric patients.

*[2] An industry-sponsored clinical trial with Prochymal® for pediatric patients.

*[3] Latest industry-sponsored clinical trial with Ryoncil® for pediatric patients.

*[4] An industry-sponsored clinical trial with Alofisel® for adult patients

*[5] Long-term efficacy and safety study of Alofisel® for perianal fistulas in patients with Crohn's disease.

1.2 CLINICAL-GRADE MANUFACTURING OF MSCS

1.2.1 MSC CULTURE PARAMETERS

Clinical-grade MSC cultures utilized for immunomodulatory applications are most often isolated from BM. The MSC culture originates from only a few cells, and therefore the cells must be expanded extensively *ex vivo* in order to obtain satisfactory cell numbers for clinical use (Ringdén et al. 2006; Bartmann et al. 2007). First, the primary culture is established by isolating the mononuclear cell fraction from the BM aspirate or other sources. MNCs are allowed to adhere for 72 hours, after which nonadherent cells are removed by washing and culturing is continued. The optimal culture medium supports the division of MSCs, but not the expansion of other residual cells, for example, macrophages.

The original number of MSCs, which gives rise to the culture, can be quantified with the CFU-F assay (Castro-Malaspina et al. 1980). The number of colony-forming units is predictive for the replicative capacity of the culture (Digirolamo et al. 1999). The age of the BM donor is crucial for the success of the culture since the number of colony-forming units has been shown to decrease with aging (Lohmann et al. 2012).

In continuous MSCs cultures, the plating density has an impact on cell yields and cell characteristics (Samuelsson et al. 2009; Sekiya et al. 2002; Bartmann et al. 2007). There are conflicting opinions about the suitable plating density for MSCs in clinical-grade cultures. However, lower seeding densities have resulted in more immature progenitor cell subsets (Sekiya et al. 2002). Prockop et al. proposed that 10 cells/cm^2 would be the optimal plating density for MSCs to maintain high numbers of the rapidly dividing cells (Prockop et al. 2001). Bartmann et al. also claimed that a low seeding density helped to maintain a high proliferative potential in MSC cultures (Bartmann et al. 2007). In the study of Doucet et al. 2005, a seeding density of 1000 cells/cm^2 produced the highest cell yields and the shortest culturing time (Doucet et al. 2005). In clinical-grade cultures, a density of 1000 cells/cm^2 was thought to be a good compromise to produce high numbers of cells (Doucet et al. 2005; Sensebé 2008; Sekiya et al. 2002).

The culture medium should be replaced partially at the beginning of culture passage to maintain the concentration of Dickkopf-1 (Dkk-1) at an adequate level. Dkk-1 is secreted by MSCs during the lag phase to facilitate entry to the cell cycle. Dkk-1 also maintains the cells in an undifferentiated state during their expansion (Gregory et al. 2003; Horwitz 2004).

Confluency in the MSC cultures should be kept under 100% during the expansion steps, and splitting of the culture at an 80-90% confluency can be

considered as optimal (Abo-Aziza and Zaki 2017). Entirely confluent cultures should be avoided since cell contact may induce senescence through a p53 and telomere-independent mechanism (Ho 2011). Contact inhibition also hinders cell proliferation by affecting transmembrane signaling and cytoskeletal reorganization, which results in senescence-associated enlarged cell morphology (Nelson 2002, Murphy 2009).

Population doubling (PD) numbers are used as a measure of aging of the cells in clinical-grade cultures. The maximum number of doublings for MSCs is 40-50 PDs in culture (Lepperdinger et al. 2008; Bieback et al. 2011). However, the methods used for counting PD numbers have not been standardized between laboratories and depend on how the start of the culture has been defined (Lepperdinger 2008, Sensebe, Prockop 2010).

1.2.2 CULTURE SUPPLEMENTS

1.2.2.1 *Fetal calf serum*

Fetal bovine serum (FBS) has been used as a universal cell culture supplement in research and the cell cultivating industry since the 1950s (Puck et al. 1958). The FBS contains essential components for cell proliferation and growth, such as hormones, vitamins, growth factors, transport proteins, and trace elements. However, the specific critical components in FBS are not known (van der Valk et al. 2010). The use of FBS involves significant lot-to-lot variation originating from seasonal and geographical variation, which leads to unexpected outcomes in laboratories (Baker 2016).

FBS is a controversial supplement in cell cultures utilized for clinical applications; the most severe concerns involve the risks to patient safety. FBS possesses a risk of animal-origin transmissible pathogens such as prions, viruses, and mycoplasma (Dormont 1999; Wessman and Levings 1999). The risk of zoonogenic infection must be considered in MSCs cultures since prions inducing transmissible spongiform encephalopathy (TSE) were demonstrated to infect murine MSCs (Cervenakova et al. 2011). FBS may also influence the patient's immune system as a xenoantigen (Selvaggi et al. 1997; Spees et al. 2004). Heiskanen et al. reported that human embryonic stem cells and MSCs cultured in an FBS supplemented medium was contaminated by N-glycolylneuraminic acid (Neu5Gc) originating from FBS (Heiskanen et al. 2007). Anaphylactic or arthus-like immune reactions have been reported with patients who have received cells cultured in FCS supplemented medium (Selvaggi et al. 1997). MSCs cultured with FBS also express a less stable transcriptional profile than MSCs cultured with other supplements and upregulate genes involved in cell cycle inhibitory control, especially with later

passage cells, which may lead to an extended lifespan (Shahdadfar et al. 2005). Regulatory authorities have strongly recommended replacing FBS with human origin culture supplements in the manufacturing of ATMPs (Bieback et al. 2019).

The developing biotechnology and pharma industry as well as increased needs for research have exacerbated the worldwide shortage of FBS, increased prices, and made the supply chain of FBS prone to fraud (Gstraunthaler et al. 2013; Van der Valk et al. 2018). In 1994, when the global amount of New Zealand serum sold for GMP manufacturing as compared to the figures of collected serum, it was observed that of the sold 30 000 L of New Zealand serum, only half had been produced in New Zealand. New Zealand serum is used in clinical-grade manufacturing since no prion-mediated BSE infection has been found in New Zealand. Another instance of fraud was uncovered in 2003 when a global supplier of FBS was caught for blending New Zealand serum with lower grade bovine serum albumin, water, and growth-promoting supplements (Gstraunthaler et al. 2013; Van der Valk et al. 2018). An investigation by the US Federal Food and Drug Administration found that during the years 2008-2013, 143 FBS batches with a total volume of 280 000 L were affected by crime (Van der Valk et al. 2018).

The use of FBS is also accompanied by ethical issues and environmental concerns since FBS is a side product of the meat and dairy industry. Animal welfare issues have also been introduced into the debate since the 800 000 L of FBS produced annually corresponds to 2 000 000 bovine fetuses (Brindley 2012).

1.2.2.2 *Platelet lysates*

Platelet-derived growth factors were shown to support the proliferation of MSCs in the study of Lucarelli et al., where the platelet-rich plasma was shown to promote the proliferation of BM stromal stem cells in a dose-dependent manner (Lucarelli et al. 2003). The first replacement of FBS was postulated in a study where human autologous plasma and allogeneic serum were used to replace FBS in MSC cultures (Shahdadfar et al. 2005). In that study, MSCs proliferated markedly better with autologous human serum than in FBS but human serum was not able to support differentiation. Allogeneic human serum supplement was reported to result in growth arrest, and cell death (Shahdadfar et al. 2005) since certain essential growth factors for MSCs are not present in human sera (Kuznetsov et al. 2000; Müller et al. 2006).

Doucet et al. suggested the use of PL supplements for *ex vivo* expansion of clinical-grade MSCs in 2005 (Doucet et al. 2005). PL prepared from platelets and plasma from various donors was shown to be a safe and efficient culture

supplement for MSCs, also supporting their differentiation potential (Doucet et al. 2005; Becherucci et al. 2018). It was claimed that PL resulted in better support for cell proliferation than FBS (Capelli et al. 2007; Doucet et al. 2005). However, one study reported that MSCs cultured with PL had a lower immunosuppressive capacity than cells cultured with FBS (Bernardo et al. 2007a). The study of Doucet and others found that the best cell yield with reduced culturing time resulted in culturing cells with 5% PL and with a density of 1000 cells/cm^2 (Doucet et al. 2005). PL was found to support the characteristics and functionality of MSCs and to be a suitable replacement for FBS in large-scale cultures (Doucet et al. 2005; Bernardo et al. 2006; Müller et al. 2006; Schallmoser et al. 2007).

Platelets are a source of growth factors and can be obtained as side-products of blood donations. Platelet products are also an ethical choice since platelet products are suitable for patient use for only up to 4-5 days but are still ideal for cell cultures (Bieback et al. 2009). Granules containing the growth-supporting factors can be released from platelets by activating the platelets with thrombin (tPRP) or through freeze-thaw cycles. However, there is no standardized practice for an optimal number of freeze-thaw cycles, and the cycles used have varied between 1-5 (Doucet et al. 2005; Laitinen et al. 2016a; Strandberg et al. 2017). However, when Strandberg et al. explored the optimal number of freeze-thaw cycles to release the maximum concentrations of growth-supporting factors, 3-5 cycles were reported as the optimum (Strandberg et al. 2017).

Platelet-derived growth factor (PDGF), transforming growth factor β1 (TGF-β1), epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) are essential mitogenic growth factors found in PL supporting MSC, chondrocyte and osteocyte proliferation (Doucet et al. 2005; Fekete et al. 2014). PDGF and bFGF are critical components of MSC survival, and blocking of PDGF-BB and bFGF resulted in a loss of proliferation (Fekete et al. 2012). The presence of the platelet-derived growth factor isoform PDGF-BB has been reported to protect MSCs from senescence initiated through the p53/p21 pathway (Zhang et al. 2016).

When platelets are lysed, they also release factors, which initiate and maintain blood clotting (Burnouf et al. 2013). Therefore, heparin or some other anti-clotting additive is used in the medium. Heparin products of non-human origin are approved medicinal products for clinical use and thus have not confronted FBS related issues.

Table 2. Examples of animal serum-free culture systems for MSCs.

Reference	Supplement	Platelet count/ml	Platelets/ml in medium	Freeze-thaw cycles	Donor pool
Shahdadfar et al. 2005	Autologous serum from whole blood	N/A	N/A	N/A	4
Doucet et al. 2005	5% Platelet lysate (PL) apheresis collection	1×10^9	0.05×10^9	2	10
Müller et al. 2006	2.5% Fresh frozen plasma (FFP)	1×10^9	25×10^6	N/A	N/A
Bernardo et al. 2007b	5% Platelet lysate (PL) apheresis collection	2.5×10^9	0.13×10^9	1	10
Lange et al. 2007	5% Platelet lysate (PL) pooled clinical products	1.5×10^9	0.08×10^9	1	7-13
Capelli et al. 2007	5% platelet lysate (PL)	1.2×10^9	0.06×10^9	2	N/A
Schallmoser et al. 2007	10% HPL; buffy coats and AB plasma	0.95×10^9 0.15×10^9	0.1×10^9	1	40 buffy coats 10 AB plasma units
(Kocaömer et al. 2007)	10% Pooled human AB serum	N/A	N/A	1	10
	10% Thrombin activated platelet-rich plasma (tPRP).	2×10^9-3×10^9	0.2×10^9-0.3×10^9	2	Several
Avanzini et al. 2009	5% platelet lysate (PL) apheresis collection	500×10^9	0.5×10^9	1	10
Castrén et al. 2015	0.5% platelet lysate with 2.5% AB plasma	300×10^9	0.8×10^9	5	4-16
Laitinen et al. 2016a	10% PRP platelet rich plasma (AB plasma)	300×10^9	0.1×10^9	2	8-52
	0.5% platelet lysate with 2.5% AB plasma	300×10^9	0.8×10^9	5	60

1.2.3 OXYGEN CONCENTRATION

The physiological oxygen concentration in the BM varies between 1-7% depending on the vascularization and distance from a sinus (Chow et al. 2001; Ito et al. 2015). Most clinical-grade MSCs are, however, cultured under atmospheric oxygen (20-21%), which exposes cells to high oxygen concentrations as compared to their physiological environment. Various studies have shown that excess oxygen, such as that to which cells are exposed during *in vitro* culturing, has been reported to cause oxidative damage and drive the cells into senescence (Burova et al. 2013; Estrada et al. 2013; Ito et al. 2015; Tsai et al. 2011).

BM-derived MSCs are especially sensitive to oxygen (Bigot et al. 2015), and many studies have reported beneficial effects of *in vitro* hypoxia on MSCs (Nold et al. 2015; Tsai et al. 2011; Jin et al. 2010). When oxygen levels during cell culturing have been set to 1-3%, a marked improvement was observed in the extension of the MSCs' lifespan and delay of senescence (Fehrer et al. 2007; Jin et al. 2010; Tsai et al. 2011), with an increase in the cell numbers being produced (Grayson et al. 2007; Nold et al. 2015; Martin-Rendon et al. 2007). *In vitro,* hypoxia has been shown to promote the proliferation of MSCs without impairing their characteristics or immunosuppressive capacity (Nold et al. 2015).

Hypoxia seems to maintain MSCs in a primary undifferentiated state and result in a higher number of CFU-F colonies throughout the lifespan of the cell culture as compared to culturing at an ambient oxygen level (Fehrer et al. 2007; Grayson et al. 2006), where the number of CFUs becomes markedly reduced over time (Schellenberg et al. 2013). Long-term exposure to *in vitro* hypoxia may, however, reduce the adipogenic differentiation markedly and inhibit entirely osteogenic differentiation (Fehrer et al. 2007). However, it seemed that both capacities could be restored by transferring the cells to ambient oxygen conditions (Fehrer et al. 2007). Short-term *in vitro* hypoxia has been shown to improve chondrogenic differentiation (Martin-Rendon et al. 2007). AT-derived MSCs also display diverse responses to lower oxygen concentrations and have different manifestations of DNA Damage-induced H2AX foci than BM-derived MSCs (Bigot et al. 2015).

Reactive oxygen species (ROS) originating from exogenous or intracellular sources induce DNA damage and thus drive cells into senescence (von Zglinicki 2002; Saretzki and von Zglinicki 1999; von Zglinicki et al. 2000). Mitochondria are essential in reducing reactive oxygen radicals, which may damage both mitochondrial (mtDNA) and cellular DNA and initiate DNA damage response (Bigot et al. 2015). *In vitro* hypoxia has been shown to favor stability and

maintenance of mtDNA after genotoxic stress, especially in BM-MSCs, and to reduce the occurrence of exogenous double strand breaks and chromosomal abnormalities (Bigot et al. 2015). According to one study, exogenous ROS were reported to promote aneuploidy in MSCs, and aneuploidy was shown to be closely associated with replicative senescence (Estrada et al. 2013).

1.2.4 REGULATORY ASPECTS

MSCs intended for clinical use are manufactured in highly regulated and controlled conditions, according to current good manufacturing practices (cGMP). MSCs are classified as ATMPs in the European Union (EU), together with products used in gene therapy and regenerative medicine (Schneider et al. 2010).

In the EU GMP guideline (Eudralex Volume 4, directive 2003/94/EC), the principles and responsibilities for ATMP manufacturing are described (Eudralex GMP guidelines Vol 4 2017). Manufacturing is supervised in EU countries by the European Medicines Agency (EMA) and in Finland by the corresponding competent authority, The Finnish Medicines Agency, Fimea. The corresponding authority in the USA is the FDA, which supervises pharmaceutical manufacturing. FDA regulations for manufacturing food and drugs have been collected on part 21 (FDA CFR part 21 2013).

The GMP and other regulatory activities aim at the safety of the patient. Regulations and recommendations have arisen from serious incidents that have occurred in the past, where careless action and non-standardized procedures have led to severe sequelae. The manufacturer must carefully characterize the factors affecting the quality and safety of the final product. These factors include the raw materials (donors/cells), process parameters and processing conditions, potency and characteristics of the product, as well as the stability during storage and delivery (Eudralex GMP guidelines Part IV 2017; FDA CFR part 21 2013).

The objective of GMP regulation is to ensure that the product is pure, safe, and efficacious. Both Eudralex and FDA guidelines recommend a risk-based approach when evaluating the safety issues and to utilize research to assess the risks; the manufacturer should expose the product to the conditions, which could reveal any unexpected quality and safety issues since several parameters may affect the characteristics and functionality of the final product (Eudralex GMP guidelines Volume 4 2017; FDA CFR part 21 2013).

1.2.5 MANUFACTURING SCALES

Cell therapy products can be manufactured on a non-industrial scale, which produces a few to multiple patient doses in one batch, depending on the culture system or on an industrial scale, where hundreds to thousands of patient doses are produced in one batch (Galipeau and Sensebé 2018). For treating a patient with GvHD, a dose of 1-2x10^6 cells per 1 kg patient body weight is often administered (Bartmann et al. 2007; Chinnadurai et al. 2017; Galipeau and Sensebé 2018). A large industry-sponsored phase III clinical trial infused 2x10^6 cells/1 kg patient body weight twice per week for four weeks (Galipeau and Sensebé 2018).

Currently, most clinical-grade MSCs are manufactured on a non-industrial scale (Naji et al. 2019). High cell yields can be obtained by upscaling the culture to bioreactors (Haack-Sørensen et al. 2018; Russell et al. 2018; Mizukami and Swiech 2018). The advantage of bioreactors is that culture conditions are highly controlled and adjustable, whereas for example, the oxygen level in planar systems such as in culture flasks or cell stacks cannot be continuously monitored and controlled (Mizukami and Swiech 2018). In a bioreactor, the culturing temperature, the proportion of dissolved oxygen, and pH in the culture can be set to an optimal level (Sensebé et al. 2013; Mizukami and Swiech 2018). In the culturing of MSCs, the bioreactor types mostly used are mono- and multilayer bioreactors, hollow fiber bioreactors, stirred tanks, or wave bioreactors, with microcarrier and perfusion systems (Mizukami and Swiech 2018). However, the challenge in the manufacturing of MSCs in bioreactors is the difficulty in detecting confluency as well as observing cell morphology, and therefore monitoring the proportion of senescent cells becomes important (Chinnadurai et al. 2017).

1.2.6 QUALITY PARAMETERS

Manufacturing of ATMPs requires aseptic processing throughout the manufacturing to ensure product sterility since the product cannot be sterilized by autoclaving or filtering. Therefore, high requirements have been set for the facilities, equipment, human resources, and controlling the manufacturing process. In-process controls and quality control analyses must be designed to measure the proper parameters to ensure the safety and potency of the product.

Testing of donors for possible viral infections is crucial for obtaining a sterile cell culture. Although regulations for manufacturing do not include consideration of the donor's age, an appropriate limit should be considered since increasing donor age exhibits a strong correlation with decreasing proliferative potential (Stolzing et al. 2008).

There are differences in opinions about the benefits of immunosuppression assays as a release criterion for product potency. A potency assay should demonstrate the biological activity of the product and be based on the intended use in the clinical application. However, there is no specific criterion for the immunosuppressive potency assay due to the complexity of MSCs' immunomodulatory mechanisms. Therefore, ISCT recommends an assay matrix including quantitative RNA and proteome analyses to complement the immunosuppression assay (Galipeau et al. 2015; Viswanathan et al. 2019).

MSCs have a limited lifespan, and because senescence is accompanied by typical changes, which may impair both the characteristics and functionality of the cells, the aging of the cells during processing should be monitored. BM-derived MSC cultures originate from a relatively small volume of BM, which contains a minor population of MSCs. The MSC culture must be expanded extensively to obtain high yields of early passage cells. During the expansion, MSCs exhaust a large proportion of their division potential, especially during the formation of the primary culture (DiGirolamo et al. 1999).

Monitoring the PD number during culturing is the primary control for cellular aging (Cristofalo et al. 1998; Wagner et al. 2010), but the implementation of a screening method for senescence would be necessary for the production process. Bertolo et al. suggested a senescence score based on evaluation of PD number, morphological, physiological and genetic markers as well as testing of senescence specific markers such as counting the proportion of senescence-associated beta-galactosidase (SA-β-gal) positive cells, and expression levels of cyclin-dependent kinase inhibitors p16 and p21 (Bertolo et al. 2016). Epigenetic changes can be analyzed for monitoring aging in clinical-grade cell cultures since aging-related methylation patterns accumulate in MSCs (Bork et al. 2010; Peffers et al. 2016).

MSCs do not seem to be prone to spontaneous transformation during culture, but chromosomal aneuploidy may occur without transformation (Tarte et al. 2010). According to one study, aneuploidy often occurs in senescent cells (Estrada et al. 2013). Genetic stability is routinely monitored from the MSC products by performing a karyotype analysis. However, the method is very inaccurate and cannot detect premalignant clonal changes (Barkholt et al. 2013).

Cryopreservation of the cells is essential to produce off-the-shelf MSC products. Cryopreservation holds a variety of benefits such as timely delivery and dosing of the product on the clinic side and better planning and control of the manufacturing. However, the risks and benefits associated with cryopreservation of MSCs have been a matter of debate since MSCs have exhibited a marked reduction of functionality after freezing and thawing. MSCs have also been reported to be cleared by the Instant Blood-mediated Immune

Reaction (IBMIR) or the complement system after thawing (François et al. 2012a; Moll et al. 2014a).

1.3 CELLULAR AGING AND SENESCENCE

Aging is a complex and continuous process starting from the embryonal development stage and continuing throughout the lifespan of the organism, resulting eventually in declining functional capabilities. At the organismal level, aging encompasses the accumulation of senescence to such an extent, where it can be observed as changes in an organism's phenotype and functionality. In addition, the likelihood of aging-related severe diseases, such as degenerative diseases or cancer, increases during the lifetime (Childs et al. 2015). At the cellular level, senescence is seen as the permanent arrest of cell division as a result of accumulating cellular damage (Hayflick 1965). The finite lifespan of mammalian cells was first described by Leonard Hayflick and co-workers in 1961 when they cultured normal diploid human fibroblasts (Hayflick and Moorhead 1961). In the senescence state, the cells lose their ability to divide but remain alive and metabolically active (Itahana et al. 2001). Hayflick and co-workers discovered the replicative senescence, which is considered as “natural aging” and described an upper limit of 50 doublings for fibroblasts (Hayflick and Moorhead 1961). Senescent cells express morphological, metabolic, and functional changes deviating from young or pre-senescent cells (Sethe et al. 2006; Kuilman et al. 2010; Mets and Verdonk 1981).

In the Hayflick model, growth arrest has been named as the M1 phase during cell culturing (Wei and Sedivy 1999). The typical characteristics of M1 are the flattened morphology and enlarged cell size as well as accumulated lysosomal content, which can be observed as increased β-galactosidase activity (Stein et al. 1999). In the M1 phase, the length of telomeres has eroded to critical lengths (Wei and Sedivy 1999). In experiments, where cell cycle regulators pRb and p53 have been inactivated, the erosion of telomeres continued together with cell division and concurrently with extensive cell death until cells enter the crisis phase M2 (Wei and Sedivy 1999). If senescence mechanisms are inactivated, and telomerase is activated, cells can bypass the crisis phase. In the crisis phase, disrupted telomere structures reveal the chromosome ends, which are repaired by the end-joining of the chromosome ends, which is followed by a continuous cycle of chromosome breakage and joining (de Lange T. 2009). A bypassed crisis phase may lead to immortalization of the cells and a malignant transformation (Wei and Sedivy 1999).

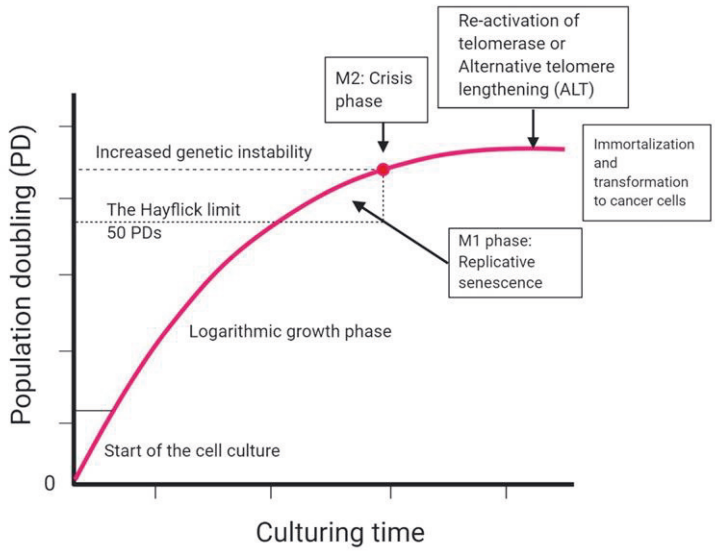

Figure 7. Hayflick model of a cell culture leading to a senescence state M1 and the crisis phase M2. After initiating the cell culture and logarithmic growth phase, when diploid somatic cells have reached the "Hayflick limit" of 50 doublings, the cells will either enter the senescence phase M1 or die if the proportion of accumulated DNA damage is high. During the M1, the telomeres have reached their critical length, and genetic instability has become increased. If mechanisms leading to senescence state (cell cycle arrest) are not functional due to inhibition or mutations, and if telomerase is re-activated, the cell may bypass the crisis phase M2 and become immortal (Wei and Sedivy 1999).

Senescence is not homogenous in a cell culture and may begin earlier in a subset of cells. However, eventually, cell cycle arrest and other senescence characteristics are synchronized as a result of a multistep process (Alcorta et al. 1996). Cells will finally die at the end of the senescence phase. Gosselin et al. showed that cells do not express apoptotic markers during senescent death, but die of high macroautophagic activity, causing the damage of cellular components (Gosselin et al. 2009).

Antagonistic pleiotropy

Senescence is essentially a mechanism to protect the organism from damage originating from replicative exhaustion, various stressors, or cancer. Damaged or aberrant cells are led to senescence before malignancy develops. However, senescence is an example of a phenomenon called antagonistic pleiotropy, where initially beneficial features can turn into mechanisms which may lead to aggravated damage in cells and tissues and finally to aging-related degenerative diseases or the development and progression of cancer (Kirkwood and Austad 2000). The main reason for the controversial nature of senescence may be caused by a senescence-associated secretory profile (SASP), where senescent

cells secrete a wide range of cytokines, chemokines, and proteolytic enzymes, which have been connected to various aging-related changes and molecules that support tumor growth (Coppé et al. 2010). Senescence can be either beneficial or detrimental, depending on the context.

1.3.1 SENESCENCE PROGRAMS

Replicative senescence is considered as natural aging, which is due to the loss of proliferative potential or replicative exhaustion of the cell and is dependent on telomere length (Hayflick and Moorhead 1961; Harley et al. 1990). The gradual shortening of telomeres and the accumulation of DNA damage lead to replicative senescence (Allsopp et al. 1992; von Zglinicki 2002; di Fagagna et al. 2003; Harley et al. 1990).

Stress-induced senescence can be triggered by various extrinsic factors such as irradiation (UV, gamma), chemotherapeutic drugs, low or high temperatures, oxidative stress, and lack of nutrients in the cell culture (Davalos et al. 2010; Toussaint et al. 2000). When cells are isolated and cultured in vitro, they are exposed to conditions differing from their natural environment such as high oxygen levels, lower temperatures, an artificial culture medium containing variable concentrations of nutrients, and a different surrounding cell environment (Sherr and DePinho 2000). One or more of these stress factors can induce a “culture shock” and trigger stress-induced senescence (Sherr and DePinho 2000).

Oncogene-induced senescence (OIS) is induced due to the activation of an oncogene (Serrano et al. 1997). Stress-induced senescence, as well as oncogene-induced senescence, are independent of telomere length (Davalos et al. 2010). Telomere length independency of stress-induced senescence has been described in mice, whose cells express telomerase continuously, in contrast to human cells (Prowse and Greider 1995).

1.3.2 MANIFESTATION OF PROGRESSED AGING AND SENESCENCE

1.3.2.1 *Cell cycle arrest*

The cell cycle is a stringently controlled process that produces two daughter cells from one cell. Cyclins are the primary regulators of the cell cycle, which in a complex with cyclin-dependent kinases (CDKs), phosphorylate the Retinoblastoma protein (pRb). CDKs are needed for the transition of the cell cycle from G1 phase to the synthesis phase, and Cyclin-CDK complexes are controlled by Cyclin-dependent kinase inhibitors (CDKIs), which consist of two

families; the INK family ($p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$, and $p19^{INK4d}$) and the Cip/Kip/Waf family including $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$ (Sherr and Roberts 1999).

$p16^{INK4a}$ (later p16), and $p21^{Cip1}$ (later p21) are the major players in the initiation of cellular senescence (Alcorta et al. 1996; Stein et al. 1999). These inhibitors act both independently and in parallel to inhibit phosphorylation of pRb (Stein et al. 1999; Ben-Porath and Weinberg 2005). Both inhibitors are used as markers for progressed aging and senescence since their expression increases markedly upon advanced aging (Krishnamurthy et al. 2004; Alcorta et al. 1996). P16 is a particularly robust biomarker and is expressed in almost all presenescent and senescent tissues (Krishnamurthy et al. 2004), except in skin cells, which do not express detectable levels of p16 (Dimri et al. 2000). Generally, p16 mRNA levels correlate strongly with protein synthesis, and protein p16 displays a strong correlation with the accumulation of β-galactosidase activity and increasing cell size (Dimri et al. 1995; Stein et al. 1999). Other p16 family proteins are not markedly expressed in aged tissues (Krishnamurthy et al. 2004).

Although p16 and p21 are both activators of senescence, the expression of p21 is regulated by a different pathway i.e. through phosphorylation of p53 (Lundberg and Weinberg 1999). The P21 level becomes rapidly elevated if cells are treated with DNA damaging agents (Stein et al. 1999) and it is also upregulated in response to many stress signals, DNA damage and oncogene activation, mediated through the tumor suppressor p53 (Stein et al. 1999; Ben-Porath and Weinberg 2005).

p16 is essential to complete the senescence state and to keep the cell cycle arrested (Stein et al. 1999). When cells are entering senescence, the levels of p21 increase faster than that of p16, and the highest expression is reached earlier. The expression of p16 is not observable in early passage cells but is elevated rapidly after the logarithmic growth phase (Alcorta et al. 1996; Stein et al. 1999; Hara et al. 1996). P21 expression declines rapidly after the initiation of senescence, while p16 remains high for at least two months to maintain cell cycle arrest (Stein et al. 1999).

1.3.2.2 *Morphological changes*

Continuously cultured cells experience dramatic changes in their morphology and size. Mets and Verdonk have described the morphological changes occurring during long term culturing in BM-derived stromal cell culture (Mets and Verdonk 1981). They identified two distinct cell morphologies from the cultures, type I representing the typical small and spindle-shaped fibroblast-like cells whereas type II were epithelial-like large cells. Early passage cells consisted

mainly of type I cells, while with passaging, the proportion of type II cells increased, and that of type I cells declined. When the culture senesced, it consisted mainly of type II cells. Prockop et al. have also identified different morphologies and proliferative properties in MSC colonies. MSCs could be grouped according to their division potential, cell size, and morphology (Prockop et al. 2001).

Many investigators have reported that senescent cells are enlarged when compared to early passage cells (Baxter et al. 2004; Stenderup et al. 2003; Mauney et al. 2004). Senescent cells show a spread morphology with filopodia and stress fibers (Mauney et al. 2004; Stenderup et al. 2003). The increase in cell size is due to the overproduction of vimentin, which causes the reorganization of intracellular intermediate filaments (Nishio and Inoue 2005). The caveolin-1 status also contributes to cell enlargement by regulating focal adhesion kinase activation, and by inducing the formation of stress fibers, lamellipodia, and filopodia (Cho et al. 2004).

1.3.2.3 *Accumulation of beta-galactosidase and lipofuscin*

The first marker used to detect senescence from cell cultures was β-galactosidase, a lysosomal enzyme capable of cleaving β-D-galactose, which is expressed at pH 6 upon cell aging (Dimri et al. 1995). Due to the strong correlation with aging, this form of β-galactosidase was termed Senescence associated β-galactosidase (SA-β-Gal), to distinguish it from another form of β-galactosidase active at pH 4-4.5 (Krishna et al. 1999). SA-β-gal is expressed by senescent fibroblasts and skin keratinocytes, but not in young or presenescent cells. It is also absent from quiescent fibroblasts, terminally differentiated keratinocytes and immortalized cells (Dimri et al. 1995). However, although SA-β-gal has been used as a general marker for senescence, it is not specific for senescence, and therefore it is insufficient to be applied as the only marker for senescence. The expression of SA-β-gal increases in immortalized cells after serum starvation, after treatment with hydrogen peroxide (Yang and Hu 2005; Dumont et al. 2000) or in confluent fibroblast culture irrespective of the cell passage (Severino et al. 2000; Yang and Hu 2005). It has been proposed that an aging-related increase in β-galactosidase activity is more likely due to the increasing of lysosomal content upon aging than the accumulation of a senescence-specific form of β-gal (Kurz et al. 2000). However, the use of β-galactosidase as a marker for senescence is a golden standard method which can be used together with other markers, nonetheless the limitations of the assay should be considered when interpreting the results. The β-galactosidase activity also reflects the *in vitro* aging but does not provide information on the *in vivo* aging of the cells.

Lipofuscin, which is a dense yellow-brown inclusion, is present in SA-β-gal positive cells (Georgakopoulou et al. 2013). Lipofuscin is formed from damaged cell components such as aggregates of oxidized proteins, lipids, and metals and is also called "the aging pigment" since it accumulates in the cytoplasm with cellular aging (Brunk and Terman 2002). Lipofuscin can be detected by fluorescence microscopy or flow cytometry due to its autofluorescent property, but it has not yet been applied widely to MSCs (Georgakopoulou et al. 2013). Lipofuscin accumulation correlates with increasing cell size, SA-β-galactosidase activity, and p16 expression, but not with p21 expression or telomere length. Therefore, it has been suggested that lipofuscin could be utilized in the detection of stress-induced senescence (Bertolo et al. 2019).

1.3.2.4 *Telomere attrition*

Telomeres are protective nucleoprotein structures located at the ends of chromosomes and are composed of TTAGGG tandem repeats and a guanine rich 3' overhang. Telomeric DNA is bound to a complex multiprotein structure, shelterin (or telosome), which consists of a T-loop DNA structure with several bound protein complexes (Figure 8.). Shelterin forms a protective structure and also regulates telomere length (Liu et al. 2004; de Lange 2005; Moyzis et al. 1988). Human telomere lengths vary between 4-15 kb depending on the cell type. Since telomeres shorten at every cell division, they have been considered as indicative of the cells' division history and thus of its replicative potential (Harley et al. 1990; Allsopp et al. 1992). Telomere length is controlled by telomerase activity during embryonal development and in immortal or cancer cells (Wright et al. 1996; Vaziri and Benchimol 1998).

Oxidative stress is another factor affecting telomere shortening (von Zglinicki 2002). Oxidative damage is not induced by extrinsic oxygen by itself but is caused by the reactive oxygen species produced in mitochondria (Passos et al. 2007). Telomere repeats, and especially the 3' overhang, rich in guanine bases, are highly sensitive to oxidative damage and, therefore, are susceptible to ROS-induced DNA strand breaks (Oikawa and Kawanishi 1999). The repair of oxidative damage in telomere sequences is less efficient than in the rest of the genome (Petersen et al. 1998). The study of Passos et al. showed that senescent fibroblasts had shorter telomeres, dysfunctional mitochondria, higher ROS levels, and more DNA double strand breaks than early passage fibroblasts (Passos et al. 2007).

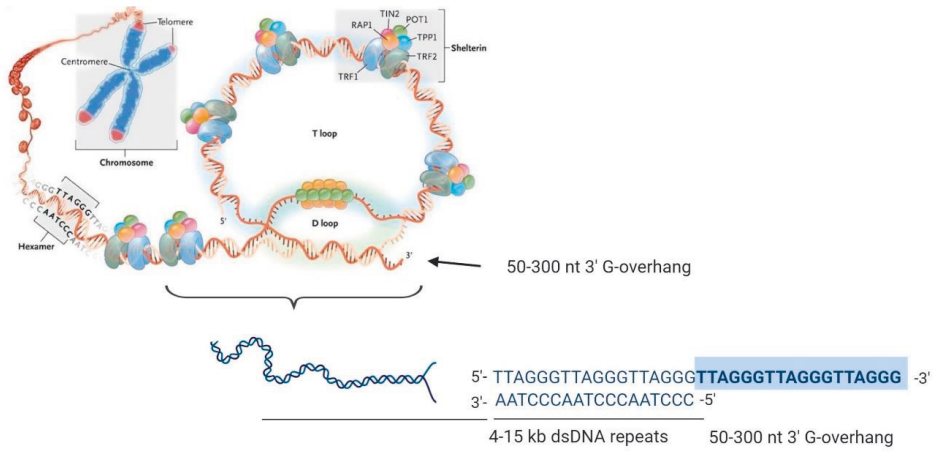

Figure 8. **Structure of human telomeres and shelterin complex.** The figure is modified from (Calado and Young 2009, O'Sullivan and Karlseder 2010).

1.3.2.5 *Epigenetic modifications*

Modifications in DNA methylation, chromatin organization, posttranslational modifications of histones, and non-coding RNAs are essential contributors to cellular aging and senescence (Franzen et al. 2016). These epigenetic changes occur at various levels and include reduced levels of the core histones, altered patterns of histone posttranslational modifications and DNA methylation, replacement of canonical histones with histone variants, and altered non-coding RNA expression (Franzen et al. 2016). Gene expression patterns may change as a response to culture conditions, but genetic changes are relatively stable (Wagner et al. 2016). Currently, the best understood and measurable aging-related epigenetic modification is the DNA methylation at specific cytosine guanine dinucleotide sites (CpG), which become either hypomethylated or hypermethylated with aging (Bork et al. 2010).

1.3.2.6 *Senescence-associated secretory profile*

One characteristic of senescent cells is that they do not proliferate but remain metabolically active. Senescent cells have undergone various metabolic changes in protein expression and secretion and express a senescence messaging secretome (Kuilman and Peeper 2009), also called the Senescence-associated Secretory Profile (SASP) (Coppé et al. 2008). The secretion of SASP factors occurs when severe DNA damage triggers senescence. Cells with SASP produce a large variety of soluble signaling factors such as cytokines, chemokines and growth factors, proteolytic enzymes, and secreted insoluble proteins and ECM

components. SASP factors enable senescent cells to modify their microenvironment, and many of them also stimulate tumor growth and progression (Coppé et al. 2010; Davalos et al. 2010).

1.3.3 AGING-RELATED ALTERATIONS IN THE CHARACTERISTICS AND FUNCTIONALITY OF MSCS

Since they are diploid somatic cells, MSCs have a restricted lifespan, where progressed aging is evident as the reduced proliferative capacity and reduced functional properties (Stenderup et al. 2003; Bieback et al. 2012; Turinetto et al. 2016). Donor's age and clinical history, as well as individual genetic variation, have been identified as major sources for the variability of MSCs in clinical use (Wu et al. 2014; Ho et al. 2008). The donor's age negatively affects the count of original CFUs and MSCs proliferative potential in culture as well as the cells' differentiation and immunosuppressive capacities (Wu et al. 2014; Banfi et al. 2000; Stenderup et al. 2003; Stolzing et al. 2008; Ross et al. 2000).

MSCs undergo approximately 30-40 PDs in cell culture (Wagner et al. 2010). Advanced aging manifests as reduced proliferation and thus lengthened doubling times (De Witte et al. 2017; Stenderup et al. 2003). MSCs undergo dramatic morphological changes upon aging as they are converted from small and spindle-shaped cells into enlarged cells with a flattened appearance with irregular shapes and granularity (Mets and Verdonk 1981; Stenderup et al. 2003). The aging-related loss of differentiation capacity has been shown in several studies; however, controversial results have been presented depending on whether all three differentiation directions are reduced or if the osteogenic or adipogenic potential is retained in some conditions (Banfi et al. 2000; Ross et al. 2000; Digirolamo et al. 1999; Bruder et al. 1997). An impaired differentiation potential upon aging seems to be, however, independent of the culture supplement used (Bieback et al. 2012).

In several reports, the immunomodulatory effects of MSCs have been reported to be markedly reduced upon aging, where a significant defect in inhibiting T-cell proliferation and cytokine secretion was seen in vitro (Chinnadurai et al. 2017; De Witte et al. 2017; Sepúlveda et al. 2014). The immunosuppressive properties are attenuated partly because of defective kynurenine production, thus indicating deficient IDO activity (Chinnadurai et al. 2017). Sepulveda et al. observed that MSCs, which failed to produce a therapeutic effect against GvHD in a clinical trial, had similarly regulated genes as senescent MSCs (Sepúlveda et al. 2014)

MSCs have a low risk of transformation to malignant cells during culture, and no tumors have been reported in clinical trials (Lalu et al. 2012). However, aneuploidy without transformation has been observed in cultured MSCs (Tarte

et al. 2010), and aneuploidy has been correlated with senescence (Estrada et al. 2012). Telomere attrition at a constant rate has been observed in most studies but has been found not to be dependent on which culture supplements were used (Bieback et al. 2009; De Witte et al. 2017). However, whether telomerase is expressed in MSC cultures remains a topic of debate. Most studies have not found telomerase expression in MSCs, but some investigators have reported telomerase activity in rat MSCs, human fetal MSCs and in one highly proliferative human BM-MSC subset (Lee et al. 2003; Fu et al. 2001; Schieker et al. 2004).

MSCs exhibit aging-related changes in their transcriptional profile (Wagner et al. 2008). One study reported 583 differently expressed genes between young and senescent cells, and another study detected over 5000 genes and 31 miRNAs that were differentially expressed in young and senescent cells (Noh et al. 2010; Wagner et al. 2008). However, there are many reports that genes regulating cell death, chromatin assembly, and vacuolization are upregulated in senescent cells, while genes involved in the cell cycle, cell growth, DNA repair, and metabolism are upregulated in young cells (Noh et al. 2010; Wagner et al. 2008).

Although MSCs are not tumorigenic *per se*, they contribute actively to the tumor microenvironment (Skolekova et al. 2016; Zhu et al. 2006). Senescent MSCs express SASP factors, which promote tumor formation and progression and particularly robust expression of SASP-related genes encoding IL-6, IL-8, MCP-2, CCL5 (Rantes), GM-CSF, MMP3 and ICAM-1 have been observed (Minieri et al. 2015; Skolekova et al. 2016; Davalos et al. 2010). With respect to the SASP factors, IL-6 is expressed at the highest levels and is known to promote tumor cell proliferation and migration (Di et al. 2014). Replicative senescent MSCs have been reported to undergo a 40-fold increase in IL-6 secretion in comparison to early passage cells (Di et al. 2014). IL-6 secreting MSCs produced large and highly vascularized tumors in a mouse xenograft model, and other studies have indicated that senescent MSCs display increased tumor cell resistance to cisplatin treatment *in vivo* (Skolekova et al. 2016; Di et al. 2014). Karnoub et al. reported that MSCs promoted metastasis in breast cancer cells and that the contributing factor between MSCs and breast cancer cells was identified as CCL5 (Karnoub et al. 2007; Davalos et al. 2010). Elsawa et al. revealed a functional correlation between the secretion of CCL5 and IL-6 (Elsawa et al. 2011)

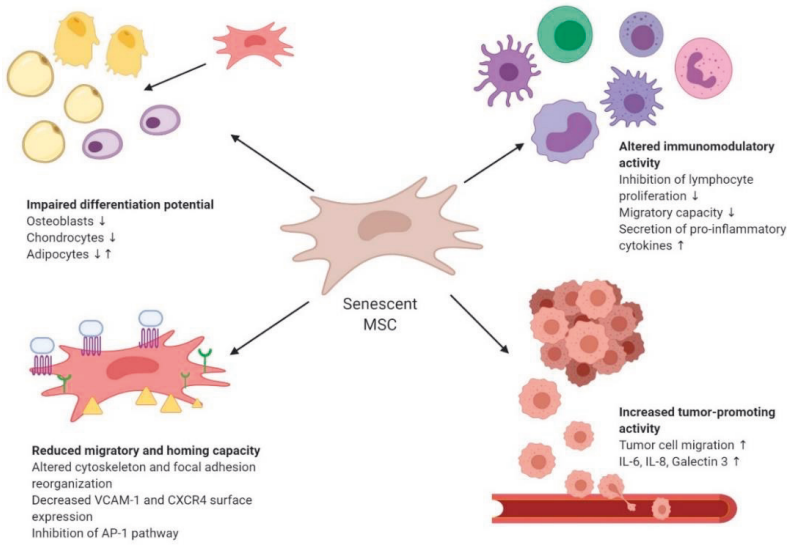

Figure 9. Functional alterations of senescent MSCs. The figure has been modified from (Turinetto et al. 2016).

1.4 CRYOPRESERVATION OF MSCS

1.4.1 CRITICAL PARAMETERS IN CRYOPRESERVATION

If one wishes to manufacture MSCs as off-the-shelf products, then the cells need to be cryopreserved. Cryopreservation of cells aims to halt cell metabolism during the storage period at extremely low (-196 - -150°C) temperatures while being able to restore cell viability and functionality after thawing. Success in cryopreservation is dependent on several critical parameters such as cooling rate, selection of a cryoprotective agent, and rate of thawing (Marquez-Curtis et al. 2015). Different cell types tolerate freezing differently; for example, embryonic stem cells are highly vulnerable to freezing, whereas adult stem cells endure cryopreservation relatively well (Hunt 2011). However, the conditions for freezing should be optimized for every cell type because cryopreservation has been associated with various forms of cellular damage (McGann et al. 1988). An optimal cooling rate is crucial for cell survival since too slow cooling results in extracellular ice formation, mitochondrial damage, high salt concentration outside the cell, and thus cell shrinkage due to osmotic stress. Too fast cooling rate results in plasma membrane damage and the formation of intracellular ice, which causes physical damage to the cell (McGann et al. 1988; Mazur 1970) (Figure 10.).

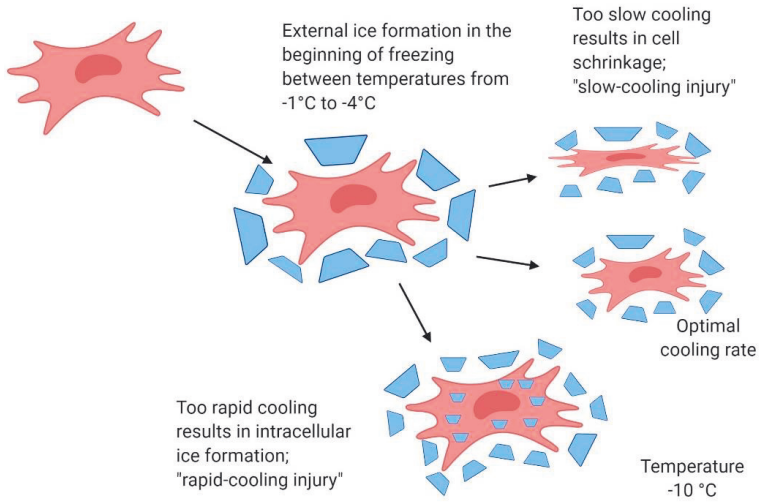

Figure 10. **Physical events in cells during freezing**. When a cell is cooled approximately to -5 °C, both intracellular and extracellular spaces are supercooled but not yet frozen. Between -5°C and -10 °C the supercooled water will flow out from the cell due to the increase in its chemical potential. If the temperature is further lowered, extracellular ice formation begins, but the intracellular space remains supercooled and unfrozen because the plasma membrane restrains the intracellular ice formation. Whether cooling injuries occur depends on the cooling rate below -10 °C. According to Mazur's two-factor hypothesis, if an optimal cooling rate is not applied, the cells will be damaged because of water's transport rates (Mazur 1970).

Freezing damages in cells are minimized by using cryoprotective agents (CPAs), which reduce formation of intracellular ice crystals by different mechanisms. The CPAs can be divided into penetrating and non-penetrating agents. Examples of penetrating CPAs are dimethyl sulfoxide (DMSO), glycerol, ethylene glycol, and propylene glycol (1,2-propanediol) (Lovelock and Bishop 1959), and examples of non-penetrating CPAs are for example sucrose, glucose, and trehalose (McGann 1978).

1.4.2 EFFECT OF CRYOPRESERVATION ON THE CHARACTERISTICS AND FUNCTIONALITY OF MSCS

Despite the use of cryoprotective agents, cells experience significant physical and biological stress during freezing. Generally, cryopreservation may result in reduced cell viability, damage to mitochondria and the plasma membrane (McGann et al. 1988). MSCs have been traditionally frozen in a 10% DMSO concentration and at a freezing rate of 1°C-5°C/min. This practice has been adopted from the freezing protocol used for hematopoietic stem cells but might not necessarily be optimal for BM-MSCs as such (Marquez-Curtis et al. 2015; Morris et al. 2016). The MSC source also influences cell survival after freezing.

For example, AT-derived MSCs tolerate freezing better than BM-MSCs (Marquez-Curtis et al. 2015).

Freezing of MSCs alters the functionality of the cells both *in vitro* and *in vivo*. The poor immunomodulatory performance of MSCs in the first major phase III clinical trial conducted by Osiris Therapeutics (Martin et al. 2010; Allison 2009; Kebriaei et al. 2019) has been hypothesized to be due to use of cryopreserved instead of fresh cells (Galipeau 2013). In MSCs, freezing and thawing result in a disruption of the actin cytoskeleton, which affects their engraftment and homing ability (Chinnadurai et al. 2014b). Reduced engraftment and homing after the systemic injection may also be due to the use of DMSO as a CPA, which may also cause the enhanced clearance of the cells by IBMIR (Moll et al. 2014a; Hoogduijn et al. 2016; Moll et al. 2016).

Thawed MSCs contain a larger proportion of apoptotic cells than a product composed of fresh cells. Cryopreserved MSCs are susceptible to T-cell mediated apoptosis compared to fresh cells (Chinnadurai et al. 2016). Exposure to T-cell mediated killing might be due to alterations in the structure of the plasma membrane, intracellular pH, or mitochondrial function (Chinnadurai et al. 2016). Frozen and thawed MSCs have a reduced ability to suppress T cell proliferation *in vitro* correlating with a deficient activity of IDO enzyme after thawing (François et al. 2012a; Chinnadurai et al. 2014a; Moll et al. 2014a). The deficient IDO activity is caused by the heat shock response induced by freezing and thawing (François et al. 2012a).

Freezing has been suggested to damage membrane proteins, which are glycosylated after translation, and this modification facilitates the recognition of MSCs by the complement system after systemic injection (Yong et al. 2017; Moll et al. 2014a). Mitochondrial injury results in accumulated oxidative stress, which in turn damages DNA and especially telomeres and thus accelerates aging (Pollock et al. 2015; Honda et al. 2001).

High donor age and increased *in vitro* age of MSCs before freezing affected the post-thaw viability and functionality negatively in the study conducted by Pollock et al. (Pollock et al. 2015). However, Andrzejewska et al. found that donor age did not affect the morphology, growth kinetics, gene expression profiles, pro-angiogenic, or immunosuppressive potential or the trilineage differentiation capacity of biobanked MSCs (Andrzejewska et al. 2019).

It has been suggested the optimal way to restore the functionality of MSCs after thawing, could be either pre-licensing the cells with IFN-γ before freezing or by recovering the cells in 24-hour culture after thawing (François et al. 2012a; Chinnadurai et al. 2016). Pre-licensing has been shown to improve the immunosuppressive properties of MSCs but not to rescue the homing ability of the cells to the lungs (Chinnadurai et al. 2014a). François et al. showed that

recovery of MSCs with a short culture period with or without IFN-γ priming after thawing restored the immunosuppressive capacity of MSCs after the heat shock response (François et al. 2012a).

A reduction in the DMSO concentration used for MSC cryopreservation did not, however, affect the T-cell driven apoptosis (Chinnadurai et al. 2016). Another study, which explored the effects of DMSO-free osmolyte-based freezing medium formulation consisting of sucrose, glycerol or isoleucine, prevented the disruption of the actin cytoskeleton, promoted cryoprotective gene expression, and modulated the CpG epigenome (Pollock et al. 2017).

2 AIMS OF THE STUDY

This study aimed:

1. To establish an optimal animal serum-free culture system for clinical-grade MSC production (I, II)

2. To compare the osteogenic differentiation capacity of MSCs in animal serum-free and fetal bovine serum supplemented 2D- and 3D-culture systems (I).

3. To examine the aging of MSCs in the established animal serum-free clinical-grade culture system (II, III, IV).

4. To develop an imaging-based method to screen cellular aging from clinical-grade MSC cultures, and to study the correlation of morphological changes to senescence-associated markers (III).

5. To study the utilization of additional freezing steps in the clinical-grade manufacturing of MSCs and to evaluate the potential effects on the quality attributes and functionality of the product (IV).

3 MATERIALS AND METHODS

3.1 ETHICS

This study and all donor protocols were approved by the Ethical Committee of the Hospital District of Helsinki and Uusimaa, Finland. Human BM-MSCs were collected from healthy volunteer donors after written informed consent.

3.2 METHODS

3.2.1 ESTABLISHMENT OF THE PRIMARY CULTURES AND CULTURE EXPANSION OF THE MSCS

All MSC primary cultures were established from BM-derived mononuclear cell (BM-MNC) fractions, which were isolated from 20-40 ml bone aspirates collected from healthy volunteer donors. Mononuclear cell fractions were isolated by Ficoll-Paque density gradient centrifugation after which the cells were seeded at a density 400 000 cells/cm^2 on 55 cm^2 plates (I) or 75 cm^2 (II-IV) flasks and were incubated for 72 hours at +37°C/5% CO_2 in an ambient oxygen concentration, except in publication II, were MSCs were cultured in 3% and ambient oxygen.

After a 72-hour incubation period, non-adherent cells were removed by careful washing, and the culture medium was changed. Culturing of the primary passage was continued until the cultures reached 80% confluence. In the subsequent passages, MSCs were seeded at a density of 1000 cells/cm^2. At every passaging, MSC cultures were washed with DPBS and detached with either Trypsin-EDTA (I) or TrypLE CTS (II-IV). Upon culturing, the culture medium was changed twice every week, and passages were detached when confluence reached 80-90%. The cells were characterized according to the ISCT criteria (Dominici et al. 2006).

For the colony-forming unit assay (CFU-F), MNCs were seeded onto 6-well plates (9.6 cm^2). Cells for the CFU-F assay were cultured similarly to the primary culture. Culturing was continued up to 14 days before staining with 0.1% crystal violet in ethanol. Colonies larger than 2 mm were counted to obtain the original CFU-F count in the BM aspirate. The original CFU-F count was used to calculate the population doubling number spent during the primary passage (passage 0). The population doubling number was calculated using the equation $PD=\log_2$

(N_H/N_1), where N_H is the number of harvested cells/cm^2, and N_1 is the number of seeded cells/cm^2. At the primary passage (p0), the CFU-F count from the CFU-F assay was used as the seeded cell density. Population doubling time was calculated as the length of each culture passage (days) divided by PDs attained during each passage.

3.2.1 CULTURE SYSTEMS FOR MSCS

3.2.1.1 *PL1 supplemented basal medium (II, III, IV)*

Platelet lysate 1 (PL1) was prepared according to the protocol devised by Schallmoser et al. (Schallmoser et al. 2007). For the preparation of PL1, 2-13 pooled PRP units were frozen and thawed twice, and the resulting lysate was used at 10% concentration in the culture medium (Table 2.). Each used PRP unit was produced by pooling buffy coats from four individual blood donations with one unit of AB plasma (both from Finnish Red Cross Blood Service). Residual leukocytes were removed by filtration before freezing at -20 °C and subsequent thawing in a +37 °C water bath. The basal culture medium was prepared from DMEM low glucose with GlutaMax (Life Technologies), 100 U/ml penicillin and 100 µg/ml streptomycin and with 40 IU/ml porcine heparin (Heparin LEO® 5000 IE/KY/ml, Leo Pharma, Sweden) (Table 3.). Prepared PL1 was functionally tested in MSC culture before use. When the culture system was translated into a clinical-grade process in the cleanroom, antibiotics were omitted from the culture medium. MSCs were plated at a density of 1000 cells/cm^2.

3.2.1.2 *PL2 supplemented basal medium (I, II)*

Platelet lysate 2 (PL2) was prepared from four platelet units not valid for patient use due to exceeding their expiry date (Finnish Red Cross Blood Service, Helsinki, Finland). The protocol for preparation of PL2 was modified from the protocol proposed by Doucet et al. (Doucet et al. 2005). Platelet concentrates, stored in platelet additive solution (30% SSP (MacoPharma, Langen, Germany), were collected by centrifugation and suspended in Octaplas® AB plasma (Octapharma AG, Switzerland) to a density of 300×10^9 platelets/ml. Platelets were lysed by five freeze-thaw cycles using an ultra-low freezer at -80 °C and a +37°C water bath. The lysate was then centrifuged at 3200g for 20 minutes, and the supernatant was collected. The supernatant was named as the PL2 supplement and was used at a 0.5% concentration as a culture medium supplement together with 2.5% AB plasma (Octaplas), 100 U/ml penicillin and 100 µg/ml streptomycin, and with 40 IU/ml porcine heparin (Heparin LEO®

5000 IE/KY/ml, Leo Pharma, Sweden) (Table 3.). The growth promotion efficiency of the PL2 was tested in MSC reference culture before use. Lysates promoting cell expansion, with at least with the cell numbers observed in FBS supplemented reference culture, were selected for use. The MSCs were plated at a density of 1000 cells/cm^2.

Table 3. *Composition of platelet lysates used as basal medium supplements.*

Supplement	Platelet count in the lysate/ml +additives	Freeze-thaw cycles	Concentration of supplement in the culture medium	Basal medium	Medium additives
PL1	300×10^9/ml+ AB plasma from 4 donors buffy coat (Schallmoser et al. 2007)	2	0.1×10^9/ml lysed platelets used as 10% concentration in the culture medium	DMEM low glucose with GlutaMax	10% AB plasma (FRCBS) 40 IU/ml heparin 100 U/ml penicillin* 100 µg/ml streptomycin*
PL2	300×10^9/ml + Octaplas®; pooled virus-inactivated fresh frozen plasma. Protocol modified from (Doucet et al. 2005)	5	15×10^9/ml lysed platelets used as 0.5% concentration in the culture medium	DMEM low glucose with L-glutamine or GlutaMax	2.5% Octaplas® 40 IU/ml heparin 100 U/ml penicillin 100 µg/ml streptomycin

*Penicillin and streptomycin were not used in clinical-grade MSC cultures processed in the cleanroom.

3.2.1.3 *Correspondence of the PL supplement abbreviations used in publications*

PL1 was prepared according to the protocol described in section 3.2.1.1 and in Table 3. and corresponds to the abbreviation PRP (platelet-rich plasma) used in publications III and IV. PL2 supplement was prepared as described in 3.2.1.2

and in Table 3. and corresponds to the abbreviation PLP (platelet lysate and plasma) used in publication I.

3.2.1.4 *2D and 3D cultures for osteogenic differentiation assays (I)*

For both 2D and 3D culture systems, MSCs were cultured in FBS supplemented culture medium and in PL2 supplemented medium. The basal medium used consisted of low-glucose DMEM supplemented with 100 U/ml penicillin, 100 ug/ml streptomycin (EuroClone, S.p.A. P.IVA, Italy) and 2 mM L-glutamine (EuroClone). For medium supplemented with FBS, heat inactivated FBS optimized for MSC culturing was used at a 10% concentration (StemCell Technologies, Vancouver, Canada). For animal serum-free cultures, the basal medium was supplemented with 0.5% PL2 and 2.5% AB plasma (Octaplas®, Octapharma).

For 2D cultures, the cells from p2 were seeded at a density of 5 000 cells/cm^2 onto 9.5 cm diameter (55 cm^2) cell culture plates. Osteogenic differentiation was initiated when the culture confluence reached 70%. Differentiation was continued for 28 days. For 3D cultures, 25×10^3 cells from p2 were inoculated in 75 µl of culture medium into the scaffold matrix composed of 7×7×10 mm Spongostan® collagen scaffold (Ferrosan, Søeborg Denmark) and were cultured on 24-well plates. After an adhesion period of 6 hours, 1 mL of expansion medium was added to each well. On the following day, the culture medium was replaced with the osteogenic differentiation medium, and differentiation was continued for up to 28 days.

The increase in cell number during differentiation was assessed by harvesting the 2D cultured cells with trypsin-EDTA and counted by using the Coulter Cell Counter (Beckmann Coulter Life Sciences, IN, USA). Cells cultured in the 3D system were harvested by dissolving the collagen scaffolds with collagenase B (1 mg/ml) (Roche, Mannheim, Germany) and then counting the cell numbers.

3.2.1.5 *Culturing of MSCs for image analysis (III)*

MSCs from donors 1-3 were cultured in small-scale culture format (75 cm^2 and 175 cm^2 cell culture flasks). Continuous culturing was performed in 75 cm^2 flasks, and 175 cm^2 flasks were used in passages p1, p3, p5, pSEN (senescent passages) to obtain sufficient cell numbers for the assays. Cells from donors 4-6 were cultured in a large-scale format, using 2-layer Cell Stacks (Corning) with 1272 cm^2 culturing area. Basal medium supplemented with PL1 was used as a culture medium in both culture formats (Table 3.). The cells were seeded at density 1000 cells/cm^2 in both small-scale and large-scale cultures. For the

image analysis, cells were seeded at 3000 cells/cm² in 6-well culture plates for further fixation and staining.

3.2.1.1 *Cell cultures for studying the interim freezing steps (IV)*

Cell culturing for publication IV mimicked the cell culturing procedures according to the clinical protocol as precisely as possible. Cell culturing was performed using 2-layer cell stacks (Corning). The basal medium corresponds to the clinical protocol (PL1 supplemented medium, Table 3.), with the exception that it was supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were plated at density 1000 cells/cm² and harvested when confluence in the culture was precisely 80%.

3.2.2 SUMMARY OF METHODS USED IN PUBLICATIONS I-IV

Detailed methods used in Publications I-IV are listed in Table 4.

Table 4. *Methods used in Publications I-IV.*

Method	Publication
Cell isolation methods	
Isolation of MSCs from the BM by density gradient centrifugation	I-IV
Cell culturing	
FBS supplemented culture for BM-MSCs	I, II
PL1 supplemented culture	II-IV
PL2 supplemented culture	I-II
Colony-forming unit-fibroblast assay (CFU-F)	I-IV
Characterization assays	
Osteogenic differentiation Alizarin Red von Kossa	 I II-IV
Adipogenic differentiation Oil Red O staining Sudan III staining	 I II-IV
Chondrogenic differentiation Alcian Blue staining	 II
Flow cytometric analysis of surface antigens CD14, CD19, CD45, CD73, CD34, CD90, CD105 CD13, CD29, CD44, CD49e HLA-DR HLA-ABC	 I-IV II I-IV II
Immunosuppression assay	
MSCs co-cultured with CFSE stained responder cells Flow cytometric analysis using CFSE staining	II, IV II, IV
Assessment of osteogenic differentiation in 2D and 3D matrices	
Alkaline phosphatase activity assay (Colorimetric pNPP Alkaline Phosphatase assay) Formation of collagen matrix (Sirius Red staining) Formation of a mineralized matrix (Alizarin Red staining) Analysis of calcium deposition (spectrophotometric measurement using Cresolphthalein Complexone assay) Quantitative PCR analysis of osteogenic markers	 I I I I I
Determination of cell surface area	
Image analysis using HCS CellOmics platform	III-IV
Aging biomarkers	
Quantitative analysis of SA-β-galactosidase activity Western blot analysis of the expression of $p16^{INK4a}$ and $p21^{Cip1/Waf}$ Telomeric terminal restriction fragment analysis	III III III

3.2.3 IMAGING-BASED METHOD TO DETERMINE AGING-RELATED CHANGES IN CELL MORPHOLOGY

An imaging-based method to detect and quantify aging-related morphological changes of MSCs was devised and optimized in publication III; it was also utilized in publication IV. The development and optimization of the method, sample preparation, imaging, and data analysis are described in detail below. Results from the imaging analysis are presented in the Results section.

3.2.3.1 *Sample preparation for the imaging analysis*

BM-MSCs for the study were derived from six clinical-grade cultures and were cultured in the research laboratory starting from passage one until senescence (pSEN). Samples for the imaging analysis were taken from every other passage until cells reached the senescence state defined as maintaining a less than 30% confluence for two consecutive weeks and expressing the typical morphological characteristics of senescent cells. The MSCs were seeded at a density of 3000 cells/cm^2 on 6-well plates suitable for imaging purposes and were allowed to attach and spread for 48 hours. The cells were fixed with 4% paraformaldehyde and were stored at +4°C under 0.02% sodium azide/PBS until staining and imaging. Before imaging, the cells were permeabilized first with 1% Tx-100/PBS for subsequent staining of the nuclei with 0.125 µg/ml DAPI (Sigma, MO, USA) and the cytoplasm with 1 µg/ml Cell Mask Deep Red stain (Life Technologies, USA).

3.2.3.2 *Image calibration and settings for image acquisition*

The images were acquired using a high content screening microscope (Cell Insight, Thermo Scientific, IL, USA) using a 10× objective (Olympus, Japan). A 630 nm filter for the signal acquisition was used for Cell Mask and 386 nm for DAPI. For defining settings for image calibration and image acquisition, several test MSCs were cultured, fixed, stained, and imaged according to the protocol. Calibration data was analyzed several times, and parameters for image gating were set manually after each iteration. The gating parameters were the minimum object size, the threshold signal intensity was when two objects are considered separate, and the threshold for finding an object's edge. An object was considered as a cell when it contained only one nucleus and was included entirely within the imaging field. The lowest size limit of 765 µm^2 was set to include only objects larger than the limit. Smaller objects were considered as debris after optimization. Careful optimization resulted in the final imaging protocol yielding only a few false positives and a moderate level of false negatives. The exposure time was adjusted separately for every acquisition run

because of dye bleaching, and the first imaging field was used for the adjustment. The imaging protocol was run automatically, and the image was focused after every tenth image to maintain uniform and comparable focus throughout the imaging.

3.2.3.3 *Image acquisition and data analysis*

Three to six wells of 6-well plates were imaged for the analysis resulting in 999-1998 images per channel at each run. Imaging was started from the center of the well and proceeded in a spiral-like fashion to minimize the optical distortion caused by the convexity of the well.

Collected images were analysed using Cell Omics Morphology Explorer software (v4, Thermo Scientific), and nine morphological parameters were selected for further analysis; length, width, area, and perimeter (size parameters) and perimeter to area ratio, length to width ratio, boxed frame ratio, convex hull area ratio, and convex hull perimeter ratio (shape parameters).

The imaging data was first cleaned by removing outliers by excluding the proportion of the smallest and largest measurements for every parameter. However, even after data cleaning, the distributions of the data were remarkably non-normal. To normalize the distributions for subsequent analysis, the Box-Cox transformation was applied for each parameter. Differences between the data groups (morphological parameters) were analysed by applying either one- or two-way analysis of variance (ANOVA). If the paired differences were statistically significant with more than 95% confidence, Bonferroni-corrected Student's t-test was used as a post-test. The hypothesis of two distributions of two samples being the same was tested using the Kolmogorov-Smirnov distribution test.

To determine the correlation coefficients between the cell surface area measurements (after outlier removal) and the senescence-associated markers, the Pearson correlation analysis was performed. Results of the correlation analysis were visualized by a heat map and a principal component analysis by using the R language.

3.2.4 TERMINAL RESTRICTION FRAGMENT ANALYSIS TO ASSESS TELOMERE LENGTH

Mean telomere lengths were measured by Southern blot analysis of terminal restriction fragments (TRF) (Kimura et al. 2010). Genomic DNA from the snap-

frozen cell pellets was purified using the Qiagen DNAeasy Blood and Tissue Kit (Qiagen, MD, USA) and was further precipitated with sodium acetate and ethanol. The integrity of the purified DNA was evaluated by electrophoresis on a 1% agarose gel. Telomere length analysis was performed from triplicate samples using a TeloTAGGG Telomere Length Assay Kit (Roche, Switzerland). For each sample, 2 µg of extracted genomic DNA was digested using the *RsaI* and *HinfI* enzymes and electrophoresed in a 0.8% agarose gel 125 V (4 V/cm) for 4.5 hours. The separated DNA was transferred to a positively charged nylon membrane (Roche, Switzerland) by Southern blotting using 20x saline-sodium citrate buffer (SSC), after which the transferred DNA was crosslinked at 120 µJ/cm² using UV light (UVP CL-100, UK). The blot was hybridized overnight with a Digoxigenin (DIG)-labeled telomere-specific probe (TTAGGG), which was subsequently detected using an alkaline phosphatase-labeled anti-DIG antibody and CDP-Star chemiluminescent substrate. The signal from the detection reaction was exposed to autoradiography film (GE Healthcare, WI, USA). The average length (kilobase pairs, kbp) of the telomeric terminal restriction fragments was calculated using ImageJ analysis software (National Institute of Health (NIH)) and Excel software (Microsoft, WA, USA) according to mean TRF= $\Sigma (OD_i \times L_i) / \Sigma (OD_i)$ where OD_i is optical density and L_i is the length of the TRF at position i. TRF signals between 3 and 20 kbp were used in the telomere length measurement (Kimura et al. 2010).

3.2.5 FLOW CHART OF THE FREEZING STEPS AND SAMPLE COLLECTION USED IN PUBLICATION IV

To study the impact of interim freezing steps on the basic manufacturing parameters and MSC functionality, samples were collected according to the following plan:

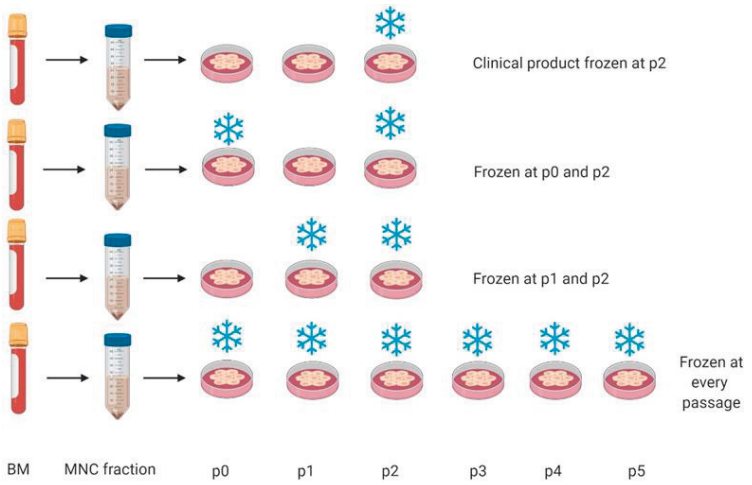

Figure 11. Sampling of the MSC cultures in the interim freezing study. According to the validated process for clinical-grade manufacturing, the cell product is frozen once and thawed just before use in the clinic. MSCs frozen either in passage 0 (primary passage) or in passage 1 and in passage 2 were compared to the culture passages of the clinical product. MSCs culture frozen at every passage were used to monitor the effects of serial freezing on proliferative potential.

Cell yield and viability were monitored at every passage as well as the PD number. Cell viability and recovery were determined after thawing at p2.

3.2.5.1 *Functionality assays*

Functionality assays such as differentiation to osteogenic and adipogenic lineages and T-cell proliferation assay were performed on the clinical product and the sample cells before and after freezing at passage 2.

In the osteogenic differentiation assay, MSCs were seeded at a density of 1000 cells/cm² on six-well cell culture plates and cultured with PL1 supplemented basal medium containing 100 IU/ml penicillin and 100 µg/ml streptomycin until 70% confluence. Osteogenic differentiation was induced with 0.1 µM dexamethasone (Dexamethasone, BioXtra, Sigma), 50 µM ascorbic acid (Ascorbic acid 2-phosphate, Sigma) and with 10 mM β-glycerophosphate disodium salt pentahydrate (AppliChem, Germany). MSCs were maintained in

the differentiation medium until the formation of visible calcium phosphate precipitate was observed. MSCs were fixed with 4% paraformaldehyde (PFA), and the calcium phosphate precipitate was stained using von Kossa staining.

For the adipogenic differentiation assay, MSCs were seeded at a density of 1000 cells/cm^2 on six-well culture plates and were cultured with PL1 supplemented basal medium with 100 IU/ml penicillin and 100 µg/ml streptomycin until 70–100% confluence. Adipogenic differentiation was induced for 3-4 days period with an induction medium consisting of αMEM GlutaMax, 10% inactivated FBS, 20 mM Hepes, 100 U/ml penicillin and streptomycin (all compounds from Gibco, Life Technologies). The medium was supplemented with the induction cocktail containing 0.1 mM indomethacin (Sigma), 44 µg/ml 3-isobutyl methyl-xanthine (IBMX-22∗), 0.5 µg/ml insulin (Insulin-0.25∗) and 0.4 µg/ml dexamethasone (DM-200∗) (∗Preadipocyte Differentiation Medium Supplement Pack, PromoCell, Italy). Control cells were only maintained in the induction medium without the induction cocktail. Adipogenic differentiation was finalized by culturing the MSCs in the terminal differentiation medium consisting of the induction medium supplemented with 0.1 mM indomethacin (Sigma), 0.5µg/ml insulin (Insulin-0.25∗) and 3.0 µg/ml ciglitazone (Ciglitazone-1.5∗) (∗Preadipocyte Differentiation Medium Supplement Pack) for 2–4 weeks until visible lipid droplets could be observed. The cells were fixed with 4% PFA and stained using Sudan III.

T-cell proliferation assay was performed using thawed PBMCs from two donors and with thawed MSCs either with a 2-4 hours recovery period in a culture or with a 24h culture recovery. Co-cultures with the CFSE labeled PBMCs and MSCs were activated using an activation cocktail containing CD3 and CD28 antibodies. An IDO inhibitor, 1-Methyl-L-Tryptophan, was used in every assay to verify IDO inhibition.

3.2.6 STATISTICAL ANALYSES

The results in publication I are presented as mean ± standard deviation. Statistical significance was evaluated with two-tailed Student's t-test, and $p<0.05$ was considered as significant. In publication II, all data was presented as mean ± standard deviation. Two-way ANOVA and Tukey's post hoc tests were used to evaluate statistical significance, $p<0.05$ was considered significant. In publication III, the statistical analyses used to analyze acquired imaging data have been described in detail in 3.2.3. due to the complexity of the analyses. The results reporting the activity of the SA-β-gal marker are presented as mean ± standard deviation. Bonferroni-corrected Student's t-test has been used to evaluate statistical significance in differences in measured β-gal activities. $P<0.05$ was considered statistically significant. In publication IV, a two-tailed paired t-test with the Mann-Whitney test was used for the comparison of two

groups. One-way ANOVA with either Bonferroni's multiple comparisons test or Brown-Forsythe test was applied when comparing multiple groups after measurement of cell surface areas. Differences were considered statistically significant when $p < 0.05$.

4 RESULTS

The results presented here are a summary of the most important findings of this study. The results are presented in a more detailed manner in the original publications.

4.1.1 PL SUPPORT OSTEOGENIC DIFFERENTIATION EQUALLY WITH FBS IN 2D AND 3D SYSTEMS (I)

In the first publication, we explored the differences in the osteogenic differentiation of MSCs between two supplements, FBS or PL2. We also investigated whether the extent of osteogenic differentiation varied between a 2D plate culture compared to differentiation performed in the 3D system, represented by a gelatin scaffold sponge. PL2 cultures in this study were not completely animal component-free, since heparin was used as a culture medium additive, and the gelatin scaffold was porcine in origin. However, both products have been approved for human medical use and are routinely used in the clinic.

When MSCs were cultured in 2D plate culture, cell numbers in PL2 supplemented culture were observed to increase 3.5-fold by day 7 after initiation of differentiation but remained constant until the end of the twenty-eight-day experiment. In FBS supplemented 2D cultures, the cells proliferated throughout the differentiation period (Figure 12A.) resulting in markedly higher total cell numbers than in PL2 supplemented cultures (FBS: 140 460 ± 17 793 cells/well and PL2: 39 000 ± 4258 cells/well, $p<0.01$). In 3D cultures, where the cells were seeded to Spongostan® matrix, equal cell numbers were counted at the end of the differentiation for both supplements (FBS; 73 993 ±17 862 cells, PL2; 78 047 ± 19 794 cells, differences statistically non-significant) (Figure 12B.).

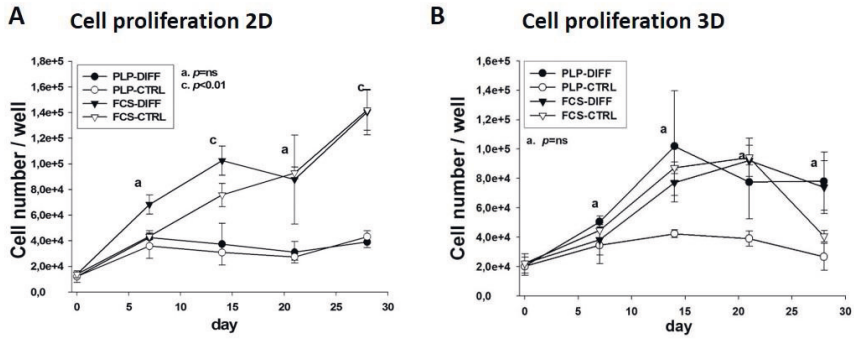

Figure 12. Cell proliferation in 2D and 3D differentiation systems. A) FBS supplemented cultures proliferated throughout the culturing, whereas PL2 supplemented 2D culture ceased to proliferate after day 7 (c. p<0.01). B) 3D differentiation system resulted in equal cell numbers for MSCs cultured with both supplements (a. difference statistically non-significant). (Castrén et al. 2015). Permission for reprint from BioMed Central, Nature Publishing.

The mRNA level of Runx2 was assessed by RT-PCR analysis of the mRNA copies. FCS supplemented the 2D plate culture showed a marked increase in the mRNA level on day 9, whereas the level of Runx2 in PL2 culture increased later, on day 14. However, 2D culture systems with both supplements resulted in almost equal levels of Runx2 on day 21 (Figure 13 A.). In 3D culture, peaks on day 9 and on day 14 were also observed for FBS and PL2 supplemented cultures, respectively, although the peaks resulted in a higher number of mRNA copies than in 2D system (Figure 13D.).

Osteocalcin (OCN), a marker for late osteogenic differentiation, was shown to increase steeply in the 2D system after day 5 for FBS and after day 14 for PL2 but resulted in a slightly higher level in PL2 supplemented cells than in cells cultured with FBS (Figure 13C.). In the 3D system, OCN levels peaked with both supplements on day 10 but were markedly higher in the FBS supplemented MSCs (Figure 13D.).

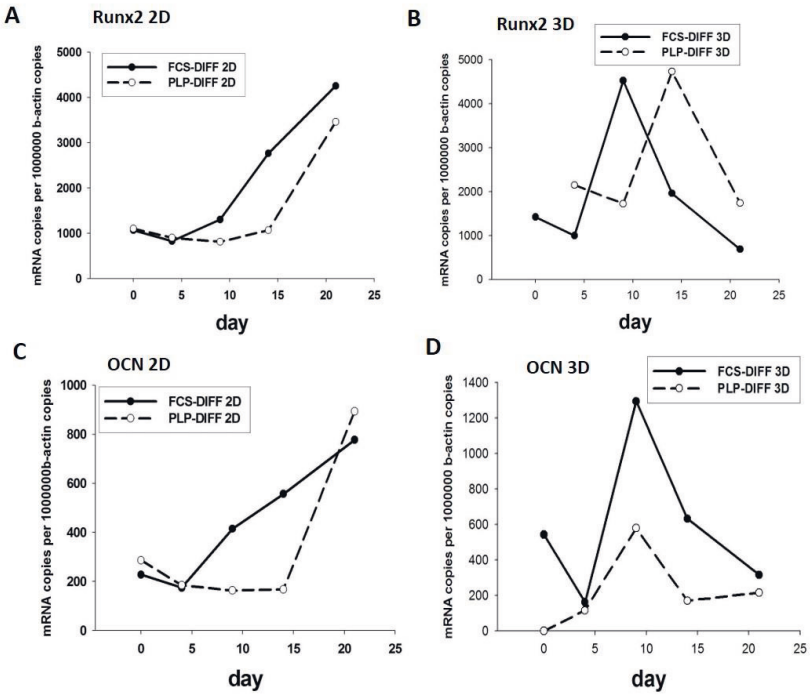

Figure 13. mRNA copies of Runx2 and OCN were determined by quantitative RT-PCR for A) Runx2 in 2D differentiation system B) Runx2 in 3D system C) OCN in 2D culture D) OCN in the 3D system. (Castrén et al. 2015). Permission for reprint from BioMed Central, Nature Publishing.

In summary, MSCs cultured with the FBS supplement showed slightly higher cell numbers during differentiation in the 2D culture system than MSCs from PL2 supplemented culture. The 3D system where cell cultures were maintained in a gelatin scaffold favored cell proliferation, and differences in cell numbers between differently supplemented cultures did not exist any longer. The determination of expression levels of osteogenic markers that are required to initiate and maintain osteogenic differentiation resulted in similar expression levels in both differentiation systems with both supplements. However, MSCs grown in FBS supplemented cultures showed earlier expression of these markers.

4.1.2 3D DIFFERENTIATION SYSTEM SUPPORTS FORMATION OF MINERALIZED MATRIX AND CALCIUM DEPOSITION WITH PL AND FBS (I)

Alkaline phosphatase (ALP), known to be an essential factor required for the initiation of osteogenic differentiation and the formation of the mineralized

matrix, was assessed by two different methods; by measuring the ALP activity using a colorimetric assay, but also by determining the number of mRNA copies by quantitative RT-PCR. MSCs in PL2 supplemented 2D cultures showed increasing ALP activity measured by the assay kit on day 4 (0.27±0.01 ng/well) compared to FBS supplemented culture (0.13±0.02 ng/well) ($p<0.01$) (Figure 14A.). Although ALP showed a high concentration for early differentiation in the PL2 cultures, the activity declined after that and subsequently remained low. In the FBS supplemented cultures, ALP activity displayed a peak on day 7 and remained high throughout the experiment (Figure 14A.). In 3D cultures, slightly higher ALP activity was observed in the PL2 cultures on day 7 than in the FBS supplemented cultures (PL2 0.012±0.003 ng/well; FBS 0.007±0.002 ng/well, difference not significant). On day 14, the ALP activity had further increased in the PL2 cultures (PL2 0.027±0.006 ng/well; FBS 0.009± 0.002 ng/well, $p<0.05$) (Figure 14B.). Quantitative RT-PCR analysis revealed a steady increase of ALP mRNA in 2D cultures with both supplements, a steeper increase was seen after day 15 (Figure 14C.). In 3D cultures, mRNA analysis showed an earlier but a modest peak of ALP mRNA in FBS supplemented culture. However, a marked increase in the expression of ALP in PL2 supplemented culture was observed on day 15, with the highest number of mRNA copies being detected on d21 (Figure 14D.).

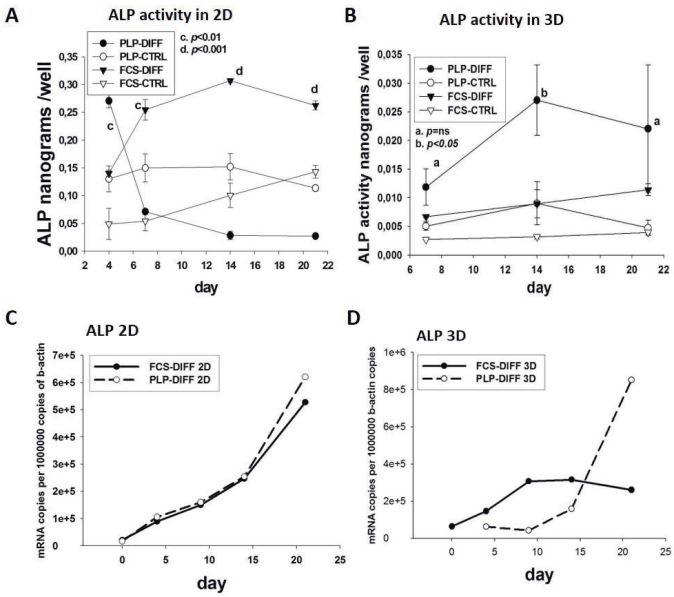

Figure 14. The activity of ALP in A) 2D system, B) 3D system for PL2, and FBS supplemented differentiation system. The number of ALP mRNA copies determined by quantitative RT-PCR analysis for C) 2D system and D) 3D differentiation systems with PL2 and FBS. (Castrén et al. 2015). Permission for reprint from BioMed Central, Nature Publishing.

The ability of differentiated MSCs to form collagen matrix and mineralized matrix was assessed by staining cells from 2D and 3D differentiation systems with Sirius Red stain for a collagen matrix and with Alizarin Red stain for a mineralized matrix. Staining was quantified by determining the optic density (OD) from the stained samples. Sirius red staining for the collagen matrix showed more intense staining for FBS in the 2D system on both analyzed days; day 14 and day 21, than for PL2 supplemented samples (Figure 15A.). When the formation of the mineralized matrix was detected in a 2D system using Alizarin Red staining, PL2 supplemented cells displayed intensive staining on days 14 and 21, and MSCs from FBS culture showed equal staining with PL2 culture on day 21 (Figure 15B.). The result was confirmed by determining the calcium deposition of the matrix by measuring the dissolved calcium concentration from the medium. The PL2 supplemented culture showed high levels of dissolved calcium in comparison to FBS culture indicating efficient calcium deposition of the mineralized matrix. Cryosection from the 3D matrix showed equal staining for PL2 and FBS supplemented cells, and the result was also confirmed by quantitation of deposited calcium level (Figure 15C. and D.).

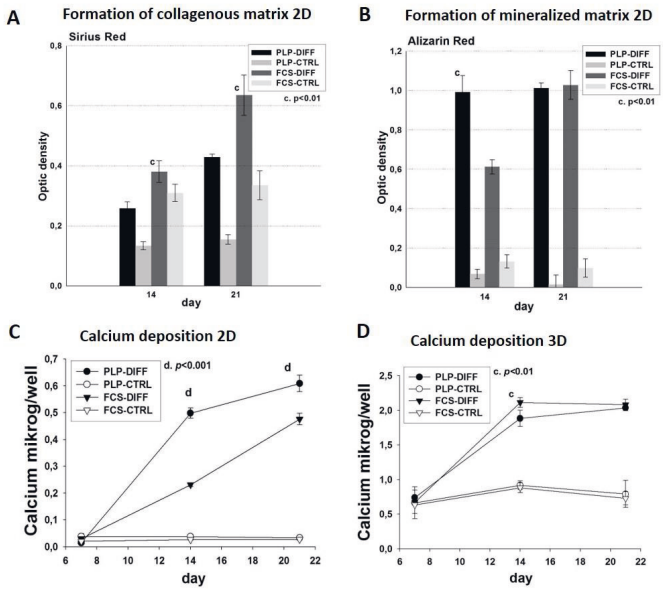

Figure 15. Detection of formation of collagen matrix and mineralized matrix from the differentiation systems. A) Sirius Red staining of MSCs from the 2D system shows more intensive staining for FBS supplemented MSCs. B) Alizarin Red staining indicates the formation of a mineralized matrix. PL2 supplemented culture shows intensive staining on both day 14 and day 21, whereas OD for FBS supplemented cells is equal to PL2 on day 21. C) Calcium deposition in the 2D system shows slightly higher deposition for PL2 culture and D) in the 3D system; both supplements show uniform deposition of calcium. The deposition level in the 3D system is higher than in the 2D system (Castrén et al. 2015). Permission for reprint from BioMed Central, Nature Publishing.

In summary, MSCs differentiated in the 3D system with PL2 resulted in a larger number of ALP mRNA copies and a higher ALP activity than FBS supplemented cells. MSCs from the PL2 supplemented 3D system also produced a mineralized matrix earlier than FBS supplemented cells and deposited calcium equally with FBS supplemented cells in the 3D differentiation system.

4.1.3 PL PROVIDES EFFICIENT SUPPORT FOR MSC PROLIFERATION UNDER AN AMBIENT OXYGEN CONCENTRATION (II)

To find the optimal culture medium supplement, which would provide good support for cell proliferation, we compared two human platelet lysate-based supplements, PL1 and PL2, to FBS. We also tested whether a lowered oxygen level (3%) would have a beneficial impact on cell proliferation and immunosuppressive properties as compared to the ambient oxygen level. PLs were prepared using two different protocols, the most noticeable difference being that platelet granules were released using two freeze-thaw cycles in PL1 while PL2 was prepared using five cycles. Culture medium compositions also varied (see Methods 3.2.1 and Table 3.).

When cells from PL1, PL2, and FBS supplemented cultures were compared, a statistically significant difference in total cell yield count could not be observed between the differently enriched cultures ($p=0.42$). However, PL1 supplemented culture produced the highest extrapolated cell yield from 20 ml BM aspirate when compared to the other supplements (Figure 16A.). Produced cell yield with PL1 under ambient oxygen was $6.31\times10^9\pm9.82\times10^9$ cells, and under 3% oxygen, the yield was $4.81\times10^9 \pm 6.78\times10^9$ cells. The numbers of cumulative PDs in PL1 supplemented MSC cultures were also higher with both oxygen concentrations when compared to the other two cultures. Cumulative PDs in PL1 cultures with 3% oxygen resulted in slightly higher PD numbers, 23.4±2.5 PD, while in ambient oxygen concentration, the PD count was 22.4±2.9 PDs. Culturing with PL1 supplement resulted in the shortest PD time, especially under 3% oxygen, where the doubling time was 1.7±0.3 days. The doubling time with PL1 in ambient oxygen was 2.1±0.5 days. The PD time was significantly shorter in cultures with PL1 than in MSCs supplemented with PL2 regardless of oxygen concentration ($p=0.015$). Still, a statistically significant difference between PL1 and FBS in PD time was not observed (Figure 17A.). To conclude, supplement PL1 resulted in a significantly shorter doubling time than PL2 supplemented MSCs. However, PL1 supplemented culture did not produce significantly higher cell numbers than the other cultures. Slightly shorter doubling times were observed with PL1 supplemented cells exposed to 3% oxygen than those exposed to ambient oxygen.

PL2 and FBS cultures originated from the same 16 BM donors and were cultured in parallel. PL1 cultures could be robustly passaged until p11 when the

culture ceased to proliferate after 46 PDs (Figure 16C.); in comparison, the PL2 supplemented culture resulted in the earliest cessation of cell proliferation at p6 after 27 PD. In these cultures, aging-related morphological changes could be observed already after passages 3 or 4, when evaluated by routine microscopy (S. Oja unpublished observations). MSCs from culture supplemented with FBS continued proliferating until p9 and 38 PDs (Figure 16D.).

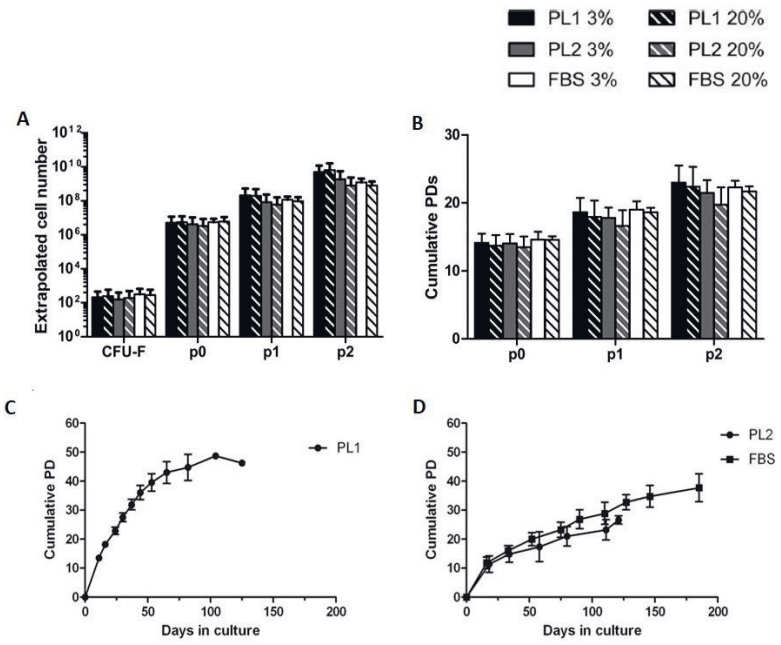

Figure 16. The effect of platelet lysates PL1, PL2, and FBS A) on cell yields and B) cumulative PD numbers in cells exposed to 3% and 20% oxygen concentrations. C) Growth kinetics of PL1 supplemented MSCs under ambient oxygen conditions D) Growth kinetics of PL2 and FBS supplemented MSCs under ambient oxygen conditions. Cell culture in Figure 16C. originate from a different donor than cells in Figure 16D., which are from the same donor and have been cultured in parallel (Laitinen et al. 2016a). Permission for reprint from Springer Nature Publishing.

To compare the functionality of the differently supplemented cells, we tested their immunosuppressive capacity in the T-cell proliferation assay. We found that PL1 supplemented MSC suppressed T-cell proliferation equally with PL2 and FBS supplemented cells (Figure 17B.). When immunosuppression of PL1 supplemented cells in different oxygen concentrations were compared, the suppressive property was slightly better in 20% oxygen than at a lower oxygen level. However, FBS supplemented cells suppressed proliferation better if cultured under *in vitro* hypoxia. Cells grown in the PL2 supplemented medium did not perform as well as PL1 or FBS in the proliferation assay, but when cultured under reduced oxygen conditions, the immunosuppressive potential of the cells cultured with PL2 was slightly improved.

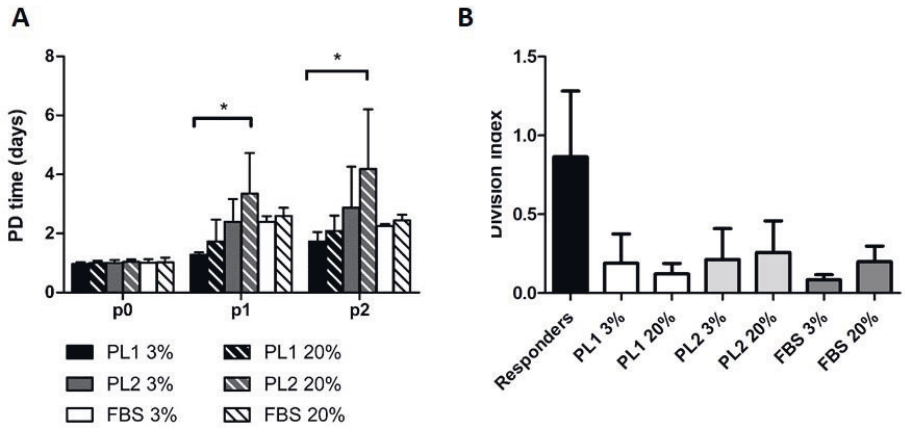

Figure 17. Impact of oxygen concentrations on MSCs A) doubling time and B) immunosuppressive properties. The oxygen concentration does not affect doubling time at the primary passage (p0), but at p1, doubling times with PL2 are markedly longer than in cultures supplemented with PL1, regardless of the oxygen concentration. PL1 and FBS does not show a statistically significant difference in doubling times. Differences in the immunosuppressive capacity between cells cultured with different supplements and in different oxygen concentrations are not statistically significant (Laitinen et al. 2016a). Permission for reprint from Springer Nature Publishing.

Thus, in summary, PL1 performs well as a supporter of cell proliferation regardless of the used oxygen levels. PL1 can produce slightly higher cell numbers within two culture passages than PL2 or FBS and within a shorter time. Hypoxia does not offer any significant benefit to MSCs cultured with PL1 in the T-cell suppression assay. PL1 cultured cells suppress T cell proliferation, as efficiently as cells that have been grown in FBS supplemented culture.

4.1.4 AGING-RELATED CHANGES IN CELL SIZE CAN BE DETECTED AND QUANTIFIED BY USING IMAGING-BASED MORPHOLOGY ANALYSIS (III)

To study aging-related changes in cell morphology, cells from six healthy donors were cultured in PL1 supplemented cultures mimicking clinical-grade MSC cultures. MSCs were cultured starting from the primary passage until the cessation of the culture. Cessated cultures showed less than 30% confluence after two weeks of culturing and displayed the typical phenotypic characteristics of senescent cells, such as flattened morphology and irregular cell shapes, and granularity. The samples for the imaging analysis and experiments detecting the expression of senescence markers $p16^{INK4a}$, $p21^{Cip1/Waf1}$, and β-galactosidase were collected from every other passage (p1, p3, p5, pSEN) until the cells reached senescence.

From the stained imaging samples, the total number of collected imaging fields was 999–1998 (examples of imaging fields are shown in Figure 18A.). Collected imaging data was processed using Cell Omics Morphology Explorer software. From the collected data, we selected nine morphology-related parameters, which would describe the aging-related changes for further analysis. The chosen parameters included both size and shape parameters. Imaging data of cell morphology was collected from 313,141 cells in total. However, before the subsequent analyses, we first cleaned the data by removing 5% of the smallest and largest values. These removed outliers were due to interpretation errors of the imaging software; in some imaging fields, debris had been interpreted as cells and overlapping cells as one large cell. After the outlier removal, the analysis was continued with 281,827 cells.

To select the morphological parameter, which could be best associated with the PD number, we performed linear fitting for each morphological parameter as an explanatory variable for the PD to a first-degree model. We also tested the donor and passage subgroup mean values and standard deviations by using them as explanatory variables. The resulting R2 coefficients for the models were highest for the size parameters such as cell area, perimeter, length, and width. When testing the standard deviations of the groups, R2 values (correlations) were even higher than the mean values, resulting in R2 values of 48% for cell area, 47% for length, 43% for perimeter, and 38% for width. Of these parameters, based on the correlations and the fact that cell area is the parameter, which is visually monitored by many operators during routine cell culturing, we selected cell surface area as the primary parameter to describe aging-related changes in cell morphology.

From the measured cell surface area values, we observed that MSCs at passages 1 and 3 were surprisingly uniform in terms of their size; the cell surface area for MSCs at p1 was 1827±329 μm^2 and the area at p3 was 2353±386 μm^2. A rapid enlargement of cell size was seen at p5 when the cell surface area had doubled as compared to p1 and reached 4198±1628 μm^2. Cell area at senescent passages (pSEN) had increased on average by 4.8-fold when compared to p1, resulting in a cell surface area of 8744±2494 μm^2 (Figure 18B.-D.). At p5, MSCs had undergone approximately 28-35 population doublings. Earlier enlargement of cell size was seen with one donor only after 25 PDs at p5 (Figure 18D.). Linear and logarithmic distributions of cell sizes show the uniform cell size at p1 and p3 but a shift towards larger cell size starting at p5. The population of smaller cells could, however, be observed also at late passages (Figure 18B.).

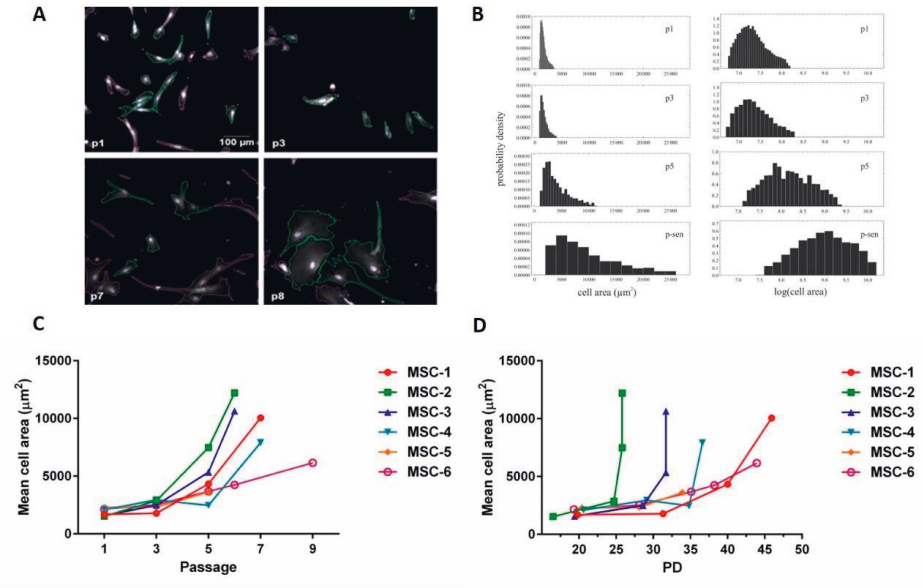

Figure 18. A.) Examples of the imaged cell cultures from passages p1, p3, p7, and p8 and recognition by the imaging software according to preset rules. Recognized and accepted cells are marked as green lines and recognized, and excluded cells are magenta lines. The correctness of the recognition was visually checked during the run, and during analysis, interpretation errors were reduced by data cleaning. B) Histograms of the distributions of cell sizes in different passages revealed a clear shift of the cell size towards enlarged phenotype at passage 5. C) When the mean cell area was plotted against culture passages (p1, p3, p5, pSEN) and D) PD number, a rapid increase in cell size could be observed, and the PD range for the increase could be determined (Oja et al. 2018). Permission for reprint from Stem Cell Research and Therapy/Nature Publishing.

4.1.5 RAPID INCREASE IN CELL SURFACE AREA CORRELATES WITH THE EXPRESSION OF SENESCENCE-ASSOCIATED MARKERS (III)

An increase in the expression of the classical senescence markers p16, p21, and β-galactosidase occurred concurrently with the increase in cell area (Figure 19.). The expression of p21 showed a pattern typical of replicative senescence; the peak in the p21 level was seen before the p16 peak, but its level rapidly decreased after p16 had reached its highest level. The highest level of expression of p16 was seen at p7, having approximately a 30-fold increase as compared to p1 (Figure 19A.). The activity of β-galactosidase increased significantly at passage 5 and continued to increase at passage 7 (for both, $p<0.001$). Constant i.e. not accelerated, telomere shortening was observed in the cell cultures during culturing (data not shown).

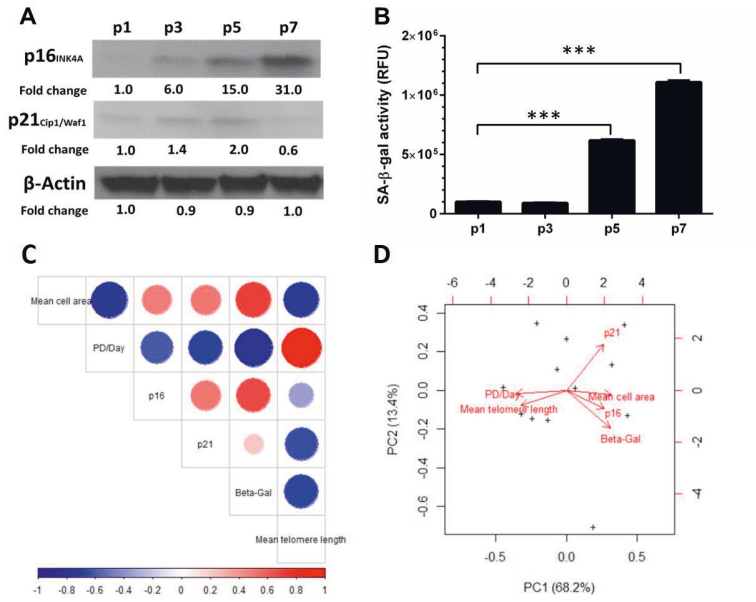

Figure 19. Correlation of senescence-associated markers with cell surface area. A) An example of a western blot analysis of the expression of cell cycle regulating cyclin-dependent kinase inhibitors, p16, and p21 from one donor. B) The activity of Senescence-associated β-galactosidase in different culture passages. The difference between p1-p3 and p5 and p7 is statistically significant $p<0.001$ C) Correlation of mean cell area with the senescence-related markers is visualized by a heat map, and D) by principal component analysis (Oja et al. 2018). Permission for reprint from Stem Cell Research and Therapy/Nature Publishing.

Visualization of Pearson correlation coefficients by the heat map and the principal component analysis reveals that the expression of p16 and β-galactosidase increase together with the cell surface area, indicating that cell proliferation had ceased and that β-galactosidase was accumulating in the cells upon culturing. These three parameters correlated negatively with the population doubling rate (PD/Day) and the mean telomere length, indicating that with progressive aging, mean telomere length had shortened, and more time was consumed in cell doubling. The expression of p21 did not seem to correlate with the other parameters in the PC analysis, however the typical expression pattern of p21 is shown in Figure 19A; a phenomenon that has also been reported by others (Alcorta et al. 1996; Stein et al. 1999), which explains the observation in the PC analysis.

4.1.6 ONE TO TWO FREEZING STEPS DO NOT ALTER THE BASIC MANUFACTURING PARAMETERS IN PASSAGE 2 MSCS (IV)

To produce the clinical product according to the manufacturing protocol at FRCBS, MSCs need to be culture-expanded until passage 2, when they are harvested and frozen. In this study, surplus cells produced during processing, at passages 0 and 1, were frozen and thawed (interim frozen cells) and further cultured until passage 2, when they were harvested and frozen again. Cell aliquots were thawed after the second freezing, and after that, viabilities and cell recovery were determined (Figure 20.).

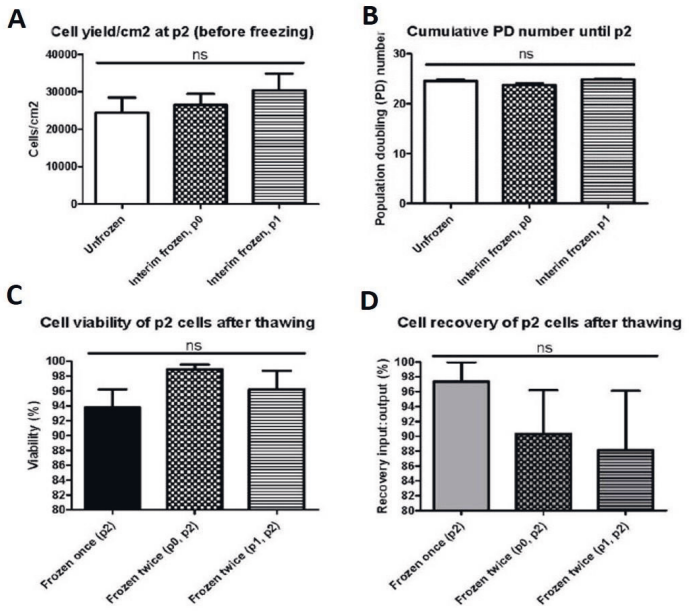

Figure 20. The effect of interim freezing steps on A) the cell yield at passage 2 before freezing, B) cumulative PD count at passage 2, C) viability after thawing at p2 and D) proportion of living cells at p2 after thawing, compared to the clinical product (cells frozen once) (Oja et al. 2019). Permission for reprint from Frontiers of Immunology/Frontiers Media.

Both cell aliquots with interim freezing steps (p0 or p1) produced slightly higher cell numbers at passage 2, as did the unfrozen cells (Figure 20A.), however, the difference in the cell yields between unfrozen cells and the interim frozen cell aliquots were not statistically significant. During the expansion, all cell aliquots underwent approximately 25 PDs until passage 2, when cells were harvested for the second freezing step (Figure 20B.). The cell viability after thawing at p2 was high, over 90% in all cell aliquots (Figure 20C.). Cell recovery after thawing at p2 can also be considered as high for all aliquots since the

recovery was over 80%, and differences between recoveries were statistically nonsignificant (Figure 20D.).

4.1.7 AGING-RELATED REDUCTION IN PROLIFERATION AND CHANGES IN MORPHOLOGY ARE NOT ACCELERATED AFTER ADDITIONAL FREEZING STEPS (IV)

MSCs, which have undergone 2 freezing steps, did not cease to proliferate earlier than the clinical product (frozen once at p2) at passages that are relevant for clinical-grade production. The control cultures that were frozen at every passage displayed a markedly diminished expansion potential, ceasing to proliferate after 35 PD. Exhaustion of the control culture was attained at passage 5, while other cell aliquots proliferated at least until passage 6. Differences in proliferation kinetics between unfrozen cells, cell aliquots frozen once (clinical product), and cells frozen twice, were not evident until p5 (Figure 21A. and 21D.). Cell aliquots, which were either unfrozen, frozen once or frozen twice, did not show any statistically significant differences in the number of cumulative PDs at passages 4 or 5. However, cells frozen at every passage had undergone significantly more PDs at these passages as compared to cell aliquots which were unfrozen, frozen once or twice ($p < 0.01$) (Figure 21B.).

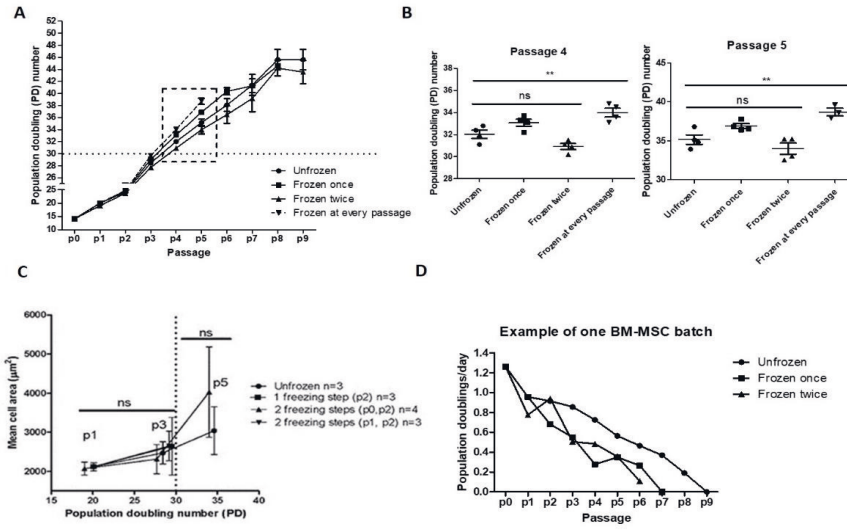

Figure 21. A) Growth kinetics from unfrozen MSC culture and cultures, which have been frozen once (clinical product), twice or at every passage. B) The difference in cumulative PD numbers between unfrozen, frozen once and frozen twice cell aliquots in passages 4 and 5 is not statistically significant. C) Cell surface areas of unfrozen MSCs and cells frozen once or twice are uniform at p1 and p3. D) An example of growth kinetics from one BM-MSC batch when unfrozen cells were compared to cell aliquots frozen once or twice (Oja et al. 2019). Permission for reprint from Frontiers of Immunology/Frontiers Media.

In these experiments, we utilized the imaging-based screening method, which was established and described in publication III, to screen for cellular aging. Due to the robust analysis and a strong correlation of changes in cell size with the expression of aging-related markers, other assays to study aging were not applied. Data from the imaging analysis indicated that cell surface areas in samples from p1-p3 remained small (2000-3000 μm^2) and uniform despite the number of freezing steps. The small size of the cells at p1 and p3 and the rapid increase in cell size at p5 indicated that MSCs at early passages did not express senescence-associated markers at significant levels yet. Evidence of variability in cell sizes began to show at p5 after 30 PDs, with cells frozen twice, showing larger cell sizes as unfrozen cells. The differences in cell surface areas between unfrozen cells and cells frozen twice were not, however, statistically significant.

4.1.8 MOST OF THE FUNCTIONALITY OF THE MSCS IS PRESERVED AFTER ONE TO TWO FREEZING STEPS (IV)

The functionality of the MSCs before freezing and after thawing at passage 2 was assessed in osteogenic and adipogenic differentiation assays (Figure 22.) and in a T-cell suppression assay (Figure 23.). We observed that osteogenic and adipogenic potentials were retained in the frozen cell aliquots and the clinical product, irrespective of the timing of the freezing steps. However, differentiation in this study was not quantified but only observed by visual examination (Figure 22.).

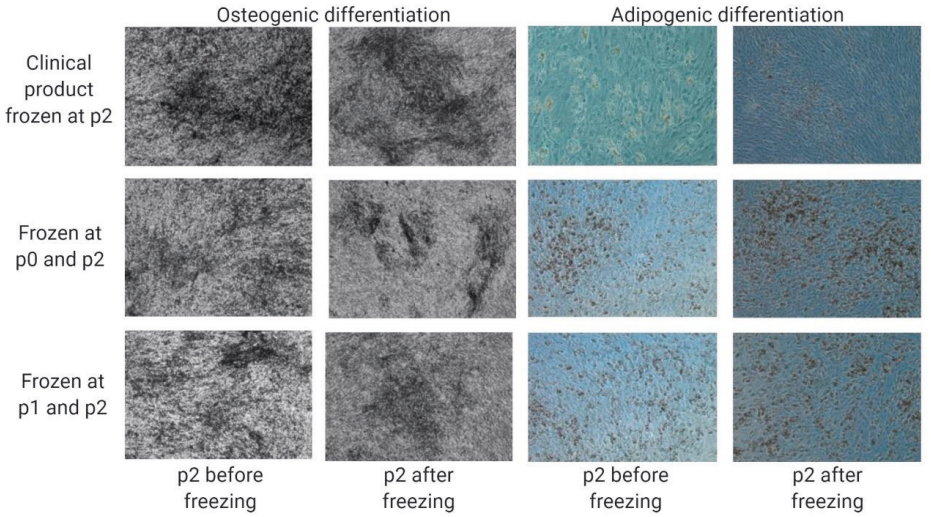

Figure 22. Osteogenic and adipogenic differentiation before and after freezing at p2. All samples show differentiation to both lineages. Differentiation analyses are quantitative and show only the occurrence of differentiation. The intensity of von Kossa and Sudan III staining is not quantified.

The ability of unfrozen and interim frozen MSCs to suppress T-cell proliferation was studied using the T-cell proliferation assay. When MSCs with one (clinical product) or two freezing steps were compared, they could both suppress T-cell proliferation (one freezing $p<0.001$, and two freezing steps p0 and p1 <0.05) (Figure 23A.). However, when the immunosuppressive properties of fresh MSCs and frozen cells were compared, an approximately 50% reduction was seen in the suppression of T -cell proliferation ($p<0.001$) (Figure 23B.).

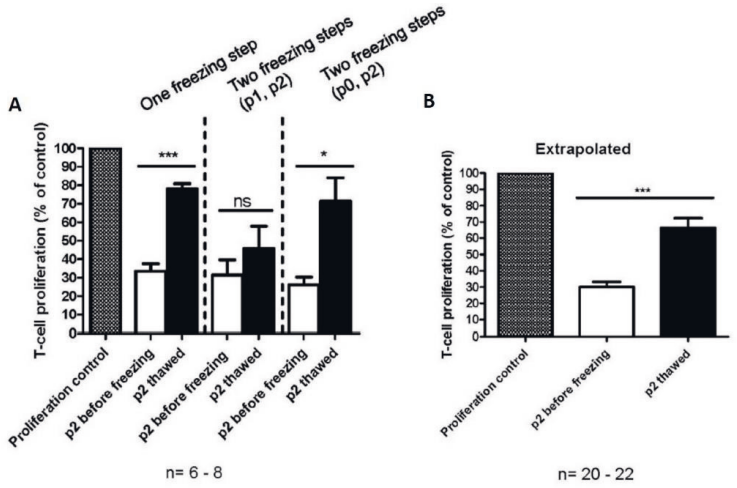

Figure 23. A) The ability of MSCs with one or two freezing steps to suppress T-cell proliferation before and after freezing at p2. B) Approximately 50% reduction in immunosuppressive capacity was seen in MSCs after freezing and thawing when compared to fresh cells (Oja et al. 2019). *p<0.005, ***p<0.001. Permission for reprint from Frontiers of Immunology/Frontiers Media.

To summarize, additional freezing steps at early passages, p0 and p1, did not alter the basic manufacturing parameters such as cell viability, cell yield, or total cumulative PD count, but showed excellent recovery and viability after thawing. Cell surface areas in unfrozen and interim frozen cells displayed uniform cell sizes, and an aging-related increase in cell sizes was seen at p5, which was not, however, the relevant passage count in our culture protocol. The small cell sizes in early passages are indicative of the absence or minor expression of aging-related markers. Osteogenic- and adipogenic differentiation assays showed comparable differentiation potential of interim frozen cells to their unfrozen counterparts. T-cell proliferation assay revealed a reduced suppression capacity of frozen and thawed cells compared to fresh cells, but the equal capacity of cells frozen once or twice to suppress T-cell proliferation.

5 DISCUSSION

This study was initiated in 2007 at a time when there was worldwide interest in MSC research and the development of clinical-grade products. Laboratories had moved on from basic research to the establishment and optimization of culture conditions for clinical-grade manufacturing. MSCs held great promise in therapeutic use, and they were thought to be the long-awaited answer to treating many immunological disorders and tissue injuries. MSC research was supported by the promising results emerging from pre-clinical experiments and the early clinical trials conducted by Lazarus and Le Blanc (Lazarus et al. 2005; Le Blanc et al. 2004). However, a major setback occurred in 2009 when an advanced phase III clinical trial (NCT00366145) for steroid refractory GvHD was performed using MSCs (Prochymal®). The results from the trial found almost no difference between the study groups (overall response, OR 82%) and placebo (OR73%) (Martin et al. 2010; Kebriaei et al. 2019).

Our study was initiated at the time when optimal culturing conditions for clinical manufacturing were being sought. Furthermore, we aimed to establish and optimize an animal serum-free culture system for the production of clinical-grade MSCs. Before the process development for clinical-grade manufacturing, we evaluated the growth and functionality supporting properties of two differently prepared PLs and compared them to FBS, which at that time was the standard supplement for cell cultures. In parallel with the testing of PLs, we determined the efficiency of osteogenic differentiation in PL supplemented and in FBS supplemented cultures, both in 2D and 3D culture systems. After establishing our facility for manufacturing, we concentrated on establishing and optimizing an imaging-based method to screen the cell products for alterations in their cell morphology, e.g. for the progressive signs of aging typical for MSCs. Monitoring of aging in the MSC cultures is a critical quality attribute since MSCs are known to lose their functionality with aging and to exhibit unwanted alterations in their characteristics and secretory profile. Finally, we sought to upscale our manufacturing process by using interim freezing steps to exploit surplus cells from previous process steps to utilize as far as possible the valuable starting material from BM aspirates received from voluntary donors.

5.1.1 PL PROVIDES AN EFFICIENT SUPPORT FOR CLINICAL-GRADE MSC CULTURES

Culture supplements PL1 and PL2 were prepared using two different protocols, which contained different numbers of freeze-thaw cycles: two cycles for PL1 (according to Schallmoser et al. 2007), and five for PL2 (modified from Doucet et al. 2005). Cultures supplemented with 10% FBS were used as controls,

because FBS supplementation had been the former standard. We found that MSCs cultured in PL1 supplemented medium could produce equal cell numbers with shorter doubling times compared to cultures with PL2 supplement or FBS, regardless of the oxygen level. PL2 supplement showed a reduced ability to support cell proliferation than PL1 or FBS. Cells produced with PL1 also showed an equal ability for suppression of T-cell proliferation as FBS supplemented cells whereas PL2 supplemented cells had a weaker ability to suppress T-cell proliferation. No marked difference was observed between ambient or low oxygen level to immunosuppression with PL1 supplemented cells, but MSCs from PL2 supplemented culture showed a slight improvement in their suppression capacity with the 3% oxygen level.

An increased proliferation rate, a decreased doubling time, delayed senescence and equally maintained functional properties of PL supplemented MSCs compared to FBS supplemented cultures have been shown in multiple studies (Doucet et al. 2005; Müller et al. 2006; Schallmoser et al. 2007; Avanzini et al. 2009; Griffiths et al. 2013). However, the composition of PL and the role of growth-promoting factors in the supplement is not entirely understood. Although most of the studies report at least similar functionality of PL to FBS, one study claimed that culturing MSCs with PL may alter the expression of some essential MSC surface molecules and impair their ability to suppress T-cell and NK-cell proliferation and cytotoxicity. That report also detected a reduced production of PGE2 but increased secretion of IL-6, IL-8, and Rantes (Abdelrazik et al. 2011). These cytokines have also been associated with the detrimental effects of the SASP profile related to progressed aging (Davalos et al. 2010; Lunyak et al. 2017).

The properties of PL may modulate the culture of MSCs in many ways; for example, the age of the blood donors has been shown to have an impact on the functionality of PL, and platelets from young donors have been reported to possess better growth-promoting properties than platelets from older donors (Lohmann et al. 2012).

An adequate number of freeze-thaw cycles to release platelet α-granules has been a topic of debate, varying from 1 to at least 4, with most of the published studies favoring 1-3 freeze-thaw cycles (Doucet et al. 2005; Kocaömer et al. 2007; Schallmoser et al. 2007; Capelli et al. 2007; Strandberg et al. 2017). We chose to test five freeze-thaw cycles in parallel with two cycles to explore the possibility of releasing a maximal concentration of growth-promoting factors. Strandberg et al. tested the release of growth factors after 1, 3, 5, 10, and 30 freeze-thaw cycles and found that the optimal number of freeze-thaw cycles seemed to be 3-5 to release selected growth factors such as isoforms of PDGF, EGF, FGF, and TGF-β1 (Strandberg et al. 2017). Our study did not, however, find support for five freeze-thaw cycles with our preparation method. We hypothesize that during PL2 preparation, some of the essential growth-

promoting factors may have been degraded, resulting in lower proliferation-promoting properties. In addition, Fekete et al. showed that inhibition of PDFG-BB, bFGF, and TGF-β1 in the culture medium resulted in a loss of cell proliferation (Fekete et al. 2012, 2014).

We observed an earlier cessation of PL2 supplemented cultures than the other cultures, and during routine microscopy, aging-related morphological changes in PL2 cultures could be seen already after p4 in some cultures. Zhang and coworkers reported that PDGF-BB protected MSCs from senescence and apoptosis and this improved their immunosuppressive properties. In their study, MSCs were isolated from patients with immune thrombocytopenia (ITP) (Zhang et al. 2015). The senescence of the ITP-MSCs was shown to be mediated through the p53/p21 pathway (Zhang et al. 2016), which can also be activated in stress-induced senescence such as that induced by suboptimal culturing conditions in a nutrient-poor culture medium (Toussaint et al. 2000). Degradation of PDGF-BB due to five freeze-thaw cycles may explain the earlier signs of senescence in PL2 supplemented MSCs. We observed a slight improvement in the proliferation of PL2 supplemented cell culture under a 3% oxygen concentration. This may indicate that the load of stress factors caused by the degradation of PDGF-BB may have been reduced in 3% oxygen and thus enabled faster proliferation.

Another deviating factor than the number of freeze-thaw cycles between the lysates was the origin of the platelets being used. PL1 was derived from blood product production where fresh platelets were suspended in fresh AB plasma, whereas PL2 was prepared from expired platelet products, which were no longer valid for patient use. The study of Jonsdottir-Buch et al. did not, however, find any difference in growth-promoting properties between lysates produced of fresh platelets and lysates where expired platelets were utilized (Jonsdottir-Buch et al. 2013). Because fresh platelet products are valuable in clinical use, expired products should be considered to be favored in the preparation of PL supplements, as proposed by Astori et al. and Jonsdottir-Buch et al. (Astori et al. 2016; Jonsdottir-Buch et al. 2013).

A large variety of methods to produce PL supplements for clinical-grade culture have been published differing in terms of the origin of the platelets, components of the supplements and techniques to release platelet α-granules (Doucet et al. 2005; Schallmoser et al. 2007; Kocaömer et al. 2007; Lange et al. 2007). Currently, there are no standardized protocols or requirements to prepare PL supplements for MSC manufacturing, and neither is there any requirement for the assay of the contents of cytokines, chemokines, or growth factors etc. We used our in-house standardized quality criteria for the used PLs with clear release criteria involving donor test results, the number of platelets, and residual leucocytes. Standardized preparation methods and defined release criteria for PL supplements should be included in practices for manufacturing

MSC products. Uniform procedures would provide better comparability between products manufactured by different facilities and would decrease the lot-to-lot variation of lysates and MSC products.

5.1.2 CULTURING OF MSCS WITH REDUCED OXYGEN DOES NOT CONFER ANY MARKED BENEFIT TO CELL EXPANSION OR FUNCTIONALITY AT LOW PASSAGE NUMBERS

The use of *in vitro* hypoxic conditions is known to improve cell proliferation and to decelerate aging of MSCs in several culture settings, since a 3% oxygen concentration is physiological for BM-MSCs (Grayson et al. 2006; Fehrer et al. 2007; von Zglinicki et al. 1995). A lower oxygen concentration during *in vitro* expansion has also been shown to improve the differentiation capacity of MSCs (Fehrer et al. 2007). In our study, it did not affect the growth-promoting performance of the PL1 whether cells were cultured under ambient oxygen or under 3% oxygen conditions. We could also not observe any statistically significant difference in the immunosuppressive capacities between the MSCs at lower the oxygen level. A lower oxygen concentration seemed, however, to slightly improve the performance of PL2, because the doubling time was markedly shorter for PL2 under 3%, than under ambient oxygen. A slight improvement in the immunosuppression was also observed with the 3% oxygen concentration; however, the difference was not statistically significant.

Grayson et al. and Estrada et al. have reported markedly improved cell expansion at lower oxygen levels as compared to culturing in a 20% oxygen concentration (Grayson et al. 2007; Estrada et al. 2012). Estarada et al. suggested that the ambient oxygen level, which is non-physiological for MSCs, causes a metabolic shift from glycolysis to oxidative phosphorylation and thus results in increased amounts of ROS in the MSC cultures. An elevated ROS level is known to affect genetic stability and telomere length in long-term culture (Estrada et al. 2012, 2013). Our study compared cell yields, doubling times, and PD numbers in the early passages, p0-02, and did not evaluate the differences in long-term cultures. Our results indicate that with the early passage numbers which are relevant to our manufacturing protocol, MSCs cultured under ambient oxygen performed equally well as cells cultured under lower oxygen concentrations. In addition, the experiments performed by Grayson et al. and Estrada et al. only detected differences between different oxygen conditions after p2 culture (Grayson et al. 2007; Estrada et al. 2012). In a plate culture system, such as ours, oxygen levels are controlled by inserting nitrogen to the incubator. However, cells are exposed to ambient oxygen during medium replacement, passaging, and harvest. Maintaining a lower oxygen concentration in a plate culture system is also a financial issue, and therefore we chose to continue our process development with MSCs cultures with an ambient oxygen level.

Controlling the oxygen level would, however, be beneficial if cell expansion were to be continued significantly further than p2. MSCs for the industry-sponsored phase II/III clinical trial with Prochymal®, were expanded until p5 (Martin et al. 2010; Prasad et al. 2011; Galipeau 2013). With these passage numbers, the utilization of closed bioreactors, with oxygen control and continuous monitoring of the metabolism, could help to improve the quality and functionality of the cell products.

Due to the ability to produce equal cell numbers with FBS culture in a shorter time, PL1 was selected for process development to establish a clinical-grade culturing process for the MSCs. Since lowering of oxygen level did not confer any significant benefit for PL1 supplemented culture, cell cultures were continued to be expanded under an ambient oxygen level using a 5-stack plate culture system.

5.1.3 PL IS AN EQUALLY GOOD SUPPORTER OF OSTEOGENIC DIFFERENTIATION AS FBS

The ability to induce differentiation of clinical-grade MSCs in an animal serum-free culture system would improve the safety of the therapy due to minimizing the risk of animal-derived pathogens and xenoimmunization reactions (Heiskanen et al. 2007; Cervenakova et al. 2011; Van der Valk et al. 2018) as well as enhancing the proliferation and differentiation of MSCs (Xia et al. 2011; Zaky et al. 2008). MSCs used for bone regeneration should retain their osteogenic properties after transplantation, or maintain their paracrine activity to secrete factors supporting bone healing (Birmingham et al. 2012). The four essential parameters for MSCs to be used for bone regeneration are 1) sufficient cell proliferation after transplantation to the target tissue, 2) the expression, and activity of alkaline phosphatase (ALP), as well as 3) the expression of other osteogenic markers and 4) the ability to produce collagen matrix able to deposit calcium (Birmingham et al. 2012). A 3D matrix together with cultured MSCs has been shown to support osteogenic differentiation and bone regeneration when compared to a matrix with fresh BM (Petite et al. 2000; Sogo et al. 2007; Kasten et al. 2008).

In our study, MSCs proliferated better in a FBS supplemented 2D plate culture than with PL2, but in the 3D gelatin matrix, both supplements supported cell proliferation equally throughout the experiment. FBS and PL2 were also found to promote equally well the osteogenic differentiation of MSCs. However, there were temporal differences in the expression of differentiation-related gene expression. In the 2D culture system, mRNA levels increased earlier in FBS supplemented cultures than in PL2. Nonetheless, both supplements eventually induced the same levels of Runx2, ALP, and OCN mRNA during 25 days of

culture. The 3D differentiation system was found to induce an earlier expression of osteogenic markers than the 2D system, during days 10-15; however, the highest ALP expression was seen on day 21 for PL2 supplemented culture. The expression levels of ALP and OCN were more robust in the 3D system than in 2D culture.

The findings of other workers confirm that PL is an effective supporter of osteogenic differentiation in MSC cultures and is comparable to FBS (Chevallier et al. 2010; Xia et al. 2011; Kasten et al. 2008; Vogel et al. 2006; Warnke et al. 2013). Chevallier et al. reported better proliferation of MSCs with PL as compared to FBS. ALP expression was found to be higher in MSCs cultured with PL. However, differences in the expression of other osteogenic markers such as Runx2 or OCN were not statistically significant between PL and FBS (Chevallier et al. 2010). The study of Kasten et al. showed that when MSCs were introduced to the 3D scaffold and were fed with freshly prepared PL, cell proliferation was significantly improved. However, no improvement in differentiation was found (Kasten et al. 2008). One of the root causes which introduced variability into the results of cell proliferation was identified as the use of different PL lots. The results reported by Warnke et al. are in agreement with our findings that PL provides an equal support for osteogenic differentiation as FBS and also that differentiation in the 3D matrix was more effective than in 2D plate culture (Warnke et al. 2013).

Bone fractures may be the type of tissue injury that is amenable to treatment with MSCs. Bone is a tissue which is able to regenerate without scarring unless the lesion is too large or bone formation has been interrupted by surgical operations (Petite et al. 2000). Autologous bone engraftment has been the primary choice for regeneration, but invasive methods to obtain the bone grafts and the risk of complications have limited the availability of this kind of therapy (Petite et al. 2000). Different methods to treat bone injuries have been exploited; autologous BM aspirate injected into the injury site, sometimes mixed with a supporting matrix before injection or mononuclear cells that have been isolated from the BM aspirate, culture-expanded and introduced into the matrix and are then engrafted to the injury site (Stanovici et al. 2016). The latter two approaches, i.e. where cells and matrix are used, have achieved the best results in experimental bone regeneration. However, the problem in cell-based grafts is the lack of vascularization. The newest applications have aimed at the vascularization of the cell-based grafts, for example, by 3D printing (Stanovici et al. 2016). However, a more important issue in tissue regeneration than the ability of MSCs to form bone may be the ability of the explants to exert paracrine effects which support bone healing in concert with osteoblast activity (Strioga et al. 2012; Almalki and Agrawal 2016).

Our study revealed that osteogenic differentiation is efficient in a 3D matrix with PL. However, we used the PL2 supplement, which was later shown to

perform poorly when compared to PL1 (Laitinen et al. 2016a). We hypothesize that the difference between FBS and PL would have been greater if PL1 should have been used as a supplement. To conclude, it is possible to achieve our aim to replace FBS as a culture supplement for MSCs intended for bone regeneration with culture expanded MSCs supplemented with PL. The osteogenic differentiation of these cells, especially when introduced to the 3D matrix, is comparable to cells grown in FBS supplemented cultures.

5.1.4 MONITORING OF CELLULAR AGING IN MSC CULTURES

Monitoring of advanced aging and senescence in MSC cultures is essential to evaluate the proliferative potential and functionality of the cells since aging is known to alter the characteristics and reduce the potency of the MSCs. In the third publication of this study, we established and optimized an imaging-based screening method to detect and quantify cell morphology and revealed that cell surface area was the best morphological parameter that described the aging-related changes. We also demonstrated that a rapid increase in cell surface area clearly correlated with the expression of classical senescence-associated markers. We succeeded in establishing a screening method that could reliably be used to detect cells expressing the primary markers for senescence, and thus we utilized this method also in our subsequent experiments.

Monitoring of MSCs *in vitro* aging and its impact on their functional properties is considered one of the cornerstones of quality control of the MSC products (Bieback et al. 2019; Menard et al. 2013; Barkholt et al. 2013). The basic measure for MSCs' aging is the determination of the cumulative PD count. We found that when the PD count was measured with our culture standards, an enlarged cell size could be detected in one sample already after 25 PDs. This sample originated from a 40-year-old donor, while the other MSCs in the study originated from donors in their early 20s. It is reasonable to hypothesize that donor age may have had an impact on the early cessation of the culture. However, the careful analysis of Andrzejewska et al. suggests that donor age does not impact on the *in vitro* aging of cultured cells (Andrzejewska et al. 2019). Our finding is, however, an important reminder that senescent cells can be found even in the MSC cultures with relatively low PD numbers. Therefore, routine monitoring of cell size should be implemented in the manufacturing process. All MSCs where cell area was quantified (publications III and IV) were derived from clinical-grade production and did not show any significant expression of senescence-related markers during the passages 1-3 (PDs 35 or lower).

In addition to counting cumulative PD numbers, changes in cell size have been known to be an indicative phenotypic characteristic of progressed aging in primary cell cultures. Elementary studies of the relationship between cell size

and morphology and the occurrence of cellular senescence were conducted by Hayflick (Hayflick and Moorhead 1961) and Mets and Verdonk (Mets and Verdonk 1981). Later, Prockop et al. and Katsube et al. separated MSCs according to their size and studied the correlation of cell size to culture characteristics and functionality (Colter et al. 2010; Katsube et al. 2008). There are also other experiments reporting that the size and morphology of the MSCs are predictive for their osteogenic and adipogenic differentiation potential, proliferative activity as well as their immunosuppressive capacity (Marklein et al. 2016; Klinker et al. 2017; Lo Surdo et al. 2013; Sasaki et al. 2014).

Our finding that a rapid increase in cell surface area displayed a strong correlation with the senescence markers is novel and opens the possibility to develop an in-process monitoring method for altered cell size further and thus to establish a first-step online screening method for senesced cells. Although there are different methods that are commonly used to study cellular aging, such as measurement of telomere length, β-galactosidase activity, or expression of cell cycle inhibitory proteins, these are not optimal for being implemented as quality control assays into clinical-grade manufacturing processes instead they are rather research tools. The senescence-associated DNA methylation signature based on six specific CpG sites (SA-DNAm), is an accurate method for detecting senescent cells from the cultures (Bork et al. 2010; Koch et al. 2012; Schellenberg et al. 2014). However, that method is laborious and may not be suitable for monitoring the culture during processing.

Imaging-based screening methods have been successfully applied to the monitoring of MSC cultures (Marklein et al. 2016; Klinker et al. 2017; Sasaki et al. 2014). These studies, where a morphology analysis has been combined with sophisticated computational methods, report that differentiation and immunosuppressive potential could be reliably predicted. Sasaki et al. described a label-free imaging method, which could be implemented into clinical-grade cell culturing without invasive handling (Sasaki et al. 2014). In addition, our approach could be further developed into a label-free method that could detect cells from images taken with a phase-contrast microscope. The detection of enlarged cells when combined with a classification analysis could be performed using deep learning-based prediction models (Sasaki et al. 2014; Chen et al. 2016; Mencattini et al. 2020). A bioreactor with a multilayer planar culture system combined with integrated cameras is already available for clinical-grade manufacturing. By utilizing these systems, screening method for senescent cells could be implemented in the large-scale manufacturing of MSCs.

5.1.5 FREEZE OR NOT TO FREEZE – HOW TO RESOLVE THE FUNCTIONALITY QUESTION?

Cryopreservation is a necessary step in producing MSCs as off-the-shelf products. The freezing step allows the storage of products and means that released products will have a well-timed distribution to the clinic. Protocols to freeze MSCs have been adopted from those techniques applied in the freezing of HSCs but these might not be optimal for MSCs (Marquez-Curtis et al. 2015). Recent reports have shown that the immunomodulatory abilities of MSCs are either abolished or markedly reduced after freezing and thawing (François et al. 2012a; Pollock et al. 2015; Moll et al. 2014a). The reason for the reduced functionality has been identified to be impaired IDO activity due to the heat shock response, although this could be rescued either by 24-48 hours culturing or by IFN-γ treatment (François et al. 2012a). Freezing may also alter the surface topology of the MSCs and thus make the cells susceptible to rejection by IBMIR and clearance by the complement system (Moll et al. 2014b, 2011). The disruption of the actin cytoskeleton structure caused by freezing and thawing has been shown to impair the ability of MSCs for engraftment and homing (Chinnadurai et al. 2014b). These freezing-related injuries may also be avoided by recovering MSCs in cell culture after thawing.

We investigated if additional freezing steps would affect the basic manufacturing parameters such as cell yield and viability, and the functional properties of MSCs. The ability to utilize additional freezing steps would provide flexibility to the manufacturing process and enable more effective production of "off-the-shelf" MSC products. Additional freezing steps would also provide a crucial possibility to create a master cell bank system and thus to produce more patient doses from the same starting material. Our results showed that two interim freezing steps did not alter the basic manufacturing parameters when compared to a clinical product which had been frozen only once in passage 2. We observed an approximately halving of the immunosuppressive capacity regardless of the number or point of the freezing steps.

In contrast to the report of Francois et al., in our experiments, MSCs' suppressive ability in T cell proliferation assay could not be improved by culture rescue (François et al. 2012a). However, when different experimental settings were used to confirm the observation, we found that minor changes in the assay protocol did change the readouts of the T-cell proliferation. This observation questions the value of this commonly used method as a measure of functionality for the clinical product.

The post-thaw functionality and practices to cryopreserve MSCs have been a topic of intensive debate, and alternatives have been sought to maintain the functionality of MSCs during cryostorage. The critical parameters in cryopreservation are the freezing rate, the cryoprotectant used, and the thawing

rate (McGann et al. 1988). Furthermore, the contact time with DMSO before freezing and after thawing is important to consider and should be maintained under one hour. Overexposure to DMSO results in the degradation of membrane integrity, impaired attachment, and altered immunophenotype (Morris et al. 2016).

Cell fitness before cryostorage should be ensured by careful monitoring of cumulative PDs and searching for signs of progressed aging since immune functionality is known to be reduced by aging due to the impairment of IDO production in MSCs (Chinnadurai et al. 2017). In addition, choice of tissue source may improve the potency of MSC products, for example, MSCs from birth-related tissues such as placenta or UC have been claimed to have a better proliferative potential and immunomodulatory properties than BM-MSCs (Jin et al. 2013; Heo et al. 2016). If MSCs are to be cultured on an industrial-scale, it is more than likely that cell products will contain some proportion of senescent MSCs, especially after five culture passages, as was the case with the MSCs cultured for the Prochymal® study (Kebriaei et al. 2009). Chinnadurai and colleagues have shown that IFN-γ treatment could revive the impaired immune functionality of MSCs due to their senescence (Chinnadurai et al. 2017). Our results demonstrate that early passage cells from young donors can be frozen during the manufacturing process, and expansion can be continued without any reduction in their viability or proliferative capacity. The findings by Moll and others also emphasized that passage 1-2 MSCs resulted in better clinical outcomes after thawing than MSCs from subsequent passages (Moll et al. 2014a).

We did not observe aging-related changes in interim frozen cells with two freezing steps. Only serial freezing with more than four freezing steps impaired cell proliferation. In addition to the mechanical stress caused by freezing, DMSO penetrates through cell membranes and leaves transient pores in the membrane (McGann et al. 1988). DMSO also alters mitochondrial integrity, which may increase the production of reactive oxygen species and thus damage encoding DNA and telomeres (Morris et al. 2016; von Zglinicki 2002; Passos et al. 2007).

Most studies that have explored the effects of freezing on MSCs have used DMSO as a cryoprotective agent. Pollock et al. found that DMSO-free freezing solution formulations improved MSCs post-thaw viability and functionality. DMSO-free formulations were also able to prevent the disruption of the actin cytoskeleton and thus improve engraftment and homing of MSCs (Pollock et al. 2017). However, Chinnadurai et al. did not observe any post-thaw improvement with a DMSO-free cryosolution (Chinnadurai et al. 2016). Pre-licensing of MSCs with IFN-γ before freezing resulted in improved inhibition of T-cell proliferation and reduced the susceptibility to lysis by activated PBMCs. Nonetheless, pre-licensed MSCs did not completely rescue the lung-homing defect caused by thawing, (Chinnadurai et al. 2016).

Several investigators have suggested that treatment with fresh MSCs seems to be the most effective approach (Chinnadurai et al. 2016; François et al. 2012a; Moll et al. 2014b; Burand et al. 2017). However, in order to be able to ensure systematic manufacturing and well-timed distribution and, most importantly, to mitigate the risks of post-thaw handling if MSCs are cultured at the clinic-side after product release, it is evident that off-the-shelf MSCs would provide the safest option for the patient. Testing of new cryosolution formulations and optimizing other crucial freezing parameters may provide new ways to improve the post-thaw functionality of MSCs.

CONCLUSIONS

In this study, we established and tested an animal serum-free culture system for obtaining clinical-grade MSCs. We evaluated the ability of two differently prepared PLs to support the proliferation and functionality of MSCs at a lowered oxygen concentration (3%) and an ambient oxygen concentration. We observed that PL1 prepared from fresh plasma and platelets using two freeze-thaw cycles could produce an equal number of MSCs as a culture supplemented with FBS, but at a shorter time. Furthermore, the ability to suppress T-cell proliferation in ambient oxygen level was comparable to FBS supplemented cells. Based on the results of that experiment, the process development for clinical-grade manufacturing of BM-MSCs was continued with PL1. Culturing of MSCs with lowered oxygen level did not confer any major benefit in the expansion or functionality of early passage MSCs, and therefore, cell culturing under an ambient oxygen concentration was also continued.

We aimed to compare the ability of MSCs to support osteogenic differentiation in 2D and 3D matrices supplemented with either FBS or PL2. We demonstrated that PL2 supplemented MSCs could support osteogenic differentiation equally well as in a FBS culture, especially in the 3D differentiation system. We observed temporal differences in the expression of osteogenic markers. However, both supplementation systems resulted in an equal expression level of osteogenic markers and similar functionality during the research period.

After the establishment of clinical-grade manufacturing for MSCs, we developed an imaging-based screening method to recognize and quantify aging-related changes in cell size. We found that MSCs in passages 1-3 were small and had a uniform at size, but a rapid increase was evident at passage 5; this occurred concurrently with a highly increased level of expression of senescence-related markers. The studied cell cultures were derived from clinical-grade cultures. Our study did not find enlarged cell sizes before p5 (35 PDs) in most of the cultures.

Finally, we explored the possibility of upscaling MSC manufacturing by using interim freezing steps during the manufacturing process, at passages p0 and p1, to obtain more products from the same starting material. We evaluated the effects of the additional freezing steps on the primary manufacturing parameters and found that when early passage cells were being processed, additional freezing steps did not alter the characteristics of the MSCs or greatly influence their functionality. However, we also found that the ability to suppress T-cell proliferation was markedly but equally reduced in the clinical product and the cell aliquots with additional freezing steps after thawing at passage 2.

In conclusion, this thesis has provided knowledge which can be utilized in developing solutions for regenerative therapy as well as for quality control, banking, and upscaling of the manufacturing process for clinical-grade MSCs.

6 ACKNOWLEDGEMENTS

This study was carried out in the Institute of Biomedicine of the University of Helsinki and in the Finnish Red Cross Blood Service in Helsinki. This study was financially supported by Väre Foundation for Pediatric Cancer Research (formerly Nona and Kullervo Väre Foundation), Finnish Red Cross Blood Service state research funding (EVO grants), and Advanced Cell Therapy Centre budget funding. This study has also gained financial support from the Foundation for Pediatric Research (Lastentautien tutkimussäätiö). I am deeply grateful for the financial possibilities to carry out this work. I especially wish to thank Chairman Sakari Arkio from the Väre Foundation for his support and encouragement.

I am grateful to the Chief Executive of the Blood Service, Professor Martti Syrjälä, Adj. Professor Jarkko Ihalainen, Adj. Professor Kari Aranko, and Professor Jukka Partanen for providing such excellent working facilities and such inspirational research colleagues. It has been a privilege to be able to work at the Blood Service.

I am deeply grateful to my thesis supervisors, Adj. Professor Matti Korhonen and Adj. Professor Johanna Nystedt and wish to express my warmest thanks for all the years we have worked together. Both of you played significant roles in my supervision; without you, this study and thesis wouldn't have been possible.

I want to warmly thank the reviewers of this thesis, Dr. Guido Moll and Adj. Professor Susanna Narkilahti, for their time and thorough revision. Their contribution was valuable and helped me to reach the next level.

I thank my thesis committee members, Professor Susanna Miettinen and Professor Elina Ikonen for their views and encouraging comments throughout the work.

I thank the Research Training Programme of Helsinki University Hospital for Children and Adolescents for the research community, training, and also for a travel grant during the first years of this study. I especially want to thank Professor Markku Heikinheimo for his support and encouragement.

I wish to express my gratitude and warmest thanks to all of my co-authors for their contributions to the publications. I thank especially Dr. Anita Laitinen and Dr. Eeva Castrén for allowing me to be a part of their projects, and Adj. Professor Antti Penttilä for his extensive expertise with the statistical analyses. His contribution was crucial to the success of the imaging study. I thank Pauno Komulainen, MD, of his contribution to setting up and optimizing the imaging

method. I also thank Dr. Tanja Kaartinen, Dr. Lotta Kilpinen, and MSc. Marja Ahti, for their valuable work and contribution to the analyses and authorship in the shared publications. I thank Adj. Professor Saara Laitinen, for her inspiration, participation, and help in integrating me into the Blood Service.

I thank Margit Sädekari, Riitta Malinen, and Piia Loponen from Blood Service for professional secretarial support. I thank Ewen MacDonald for the revision of the language of this thesis.

I thank the technical staff of the R&D Department and the Advanced Cell Therapy Centre for their excellent work; Birgitta Rantala, Sirkka Hirschovitz-Gertz, Susanna Räsänen, Iris Tallus, Satu Happonen, and Johanna Matikainen. You all are highly skilled and working with you was always both inspiring and fun. You are the best help that a researcher can have!

I thank my work community at the Department of Anatomy; Pauno, Ariel, Eeva, Heidi, Pipsa Kaipainen, Marja-Leena Piiroinen, Hanna Wennäkoski, Outi Rauanheimo and Reijo Karppinen.

I want to thank my work community at Blood Service; Ani, Erja, Johanna, Tanja, Gitta, Sirkka, Suski, Lotta A., Lotta K., and Lotta S., Jan, Annu, Pilvi, Kaarina, Joni, Sisko, Paula, Iris, Satu H., Johanna M., Ulla, Ilja, Teija, Heli and Tiia, for all the fun and inspiring moments during lunch and coffee breaks and outside the workplace. I have always had such a lovely time with you and a lot of laughs! Especially I want to thank you all for the comfort and support when my father died suddenly in 2017.

I thank my new colleagues at FinVector; Piia, Kaisa, Päivi, Lucia, and Lisette (”Lucette”), Katriina, Silja, Kati, and Maija for support and inspiring discussions. You have shared with me these last moments in the completion of the thesis. I thank Dr. Minna Karhinen and Adj. Professor Tuija Kekarainen for the encouragement to finalize my degree.

I warmly thank my close friends; Maarit, Anne, Terhi, Tanja-Maria and Satu for your friendship and support during all these years.

I thank my parents, Kristiina and Timo Franssi, and my sister Salla Franssi, for their endless love and belief in me. I dedicate this thesis to my father’s memory.

Finally, my super thanks go to my own family; my husband Joni and our beautiful and bright children, Ella and Emil. Thank you for your love and understanding during the writing of this thesis. Our children have been born and raised along with this study. It has been wonderful to see that also the next generation is interested in cells and the wonders of nature. Ella and Emil, Mommy will always love you!

Thank you, Joni, for your professional help with the imaging and statistical analyses, and for inspiring discussions about science. As a scientist yourself, you have always shown understanding about my work. In our private life, thank you for your love and support! I love you!

Kuopio, September 2020

Sofia Oja

7 REFERENCES

Abdelrazik H, Spaggiari GM, Chiossone L, Moretta L. 2011. Mesenchymal stem cells expanded in human platelet lysate display a decreased inhibitory capacity on T- and NK-cell proliferation and function. *Eur J Immunol* **41**: 3281–3290.

Abo-Aziza FAM, Zaki AA. 2017. The impact of confluence on bone marrow mesenchymal stem (BMMSC) proliferation and osteogenic differentiation. *Int J Hematol Stem Cell Res* **11**: 121–132.

Aggarwal S, Pittenger MF. 2005. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* **105**: 1815–1822.

Al-Najar M, Khalil H, Al-Ajlouni J, Al-Antary E, Hamdan M, Rahmeh R, Alhattab D, Samara O, Yasin M, Abdullah A Al, et al. 2017. Intra-articular injection of expanded autologous bone marrow mesenchymal cells in moderate and severe knee osteoarthritis is safe: A phase I/II study. *J Orthop Surg Res* **12**.

Alcorta DA, Xiong Y, Phelps D, Hannon G, Beach D, Barrett JC. 1996. Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts. *Biochemistry* **93**: 13742–13747.

Alfaifi M, Eom YW, Newsome PN, Baik SK. 2018. Mesenchymal stromal cell therapy for liver diseases. *J Hepatol* **68**: 1272–1285.

Algeri M, Conforti A, Pitisci A, Starc N, Tomao L, Bernardo ME, Locatelli F. 2015. Mesenchymal stromal cells and chronic inflammatory bowel disease. *Immunol Lett* **168**: 191–200.

Allison M. 2009. Genzyme backs Osiris, despite Prochymal flop. *Nat Biotechnol*.

Allsopp RC, Vaziri, Homayoun, Patterson, Christopher; Glodstein S, Younglai E V, Bruce Futcher A, Greidert CW, Harley CB. 1992. Telomere length predicts replicative capacity of human fibroblasts. **89**: 10114–10118.

Almalki SG, Agrawal DK. 2016. Key transcription factors in the differentiation of mesenchymal stem cells. *Differentiation* **92**: 41–51.

Álvarez-Viejo M, Menéndez-Menéndez Y, Otero-Hernández J. 2015. CD271 as a marker to identify mesenchymal stem cells from diverse sources before culture. *World J Stem Cells* **7**: 470.

Andrzejewska A, Catar R, Schoon J, Qazi TH, Sass FA, Jacobi D, Blankenstein A, Reinke S, Krüger D, Streitz M, et al. 2019. Multi-Parameter Analysis of Biobanked Human Bone Marrow Stromal Cells Shows Little Influence for Donor Age and Mild Comorbidities on Phenotypic and Functional Properties. *Front Immunol* **10**.

Arai F, Hirao A, Ohmura M, Sato H, Matsuoka S, Takubo K, Ito K, Koh GY, Suda T. 2004. Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell* **118**: 149–161.

Astori G, Amati E, Bambi F, Bernardi M, Chieregato K, Schäfer R, Sella S, Rodeghiero F. 2016. Platelet lysate as a substitute for animal serum for the ex-vivo expansion of mesenchymal stem/stromal cells: Present and future. *Stem Cell Res Ther* **7**: 1–8.

Augello A, Tasso R, Negrini SM, Amateis A, Indiveri F, Cancedda R, Pennesi G. 2005. Bone marrow mesenchymal progenitor cells inhibit lymphocyte proliferation by activation of the programmed death 1 pathway. *Eur J Immunol* **35**: 1482–1490.

Avanzini MA, Bernardo ME, Cometa AM, Perotti C, Zaffaroni N, Novara F, Visai L, Moretta A, Del Fante C, Villa R, et al. 2009. Generation of mesenchymal stromal cells in the presence of platelet lysate: A phenotypic and functional comparison of umbilical cord blood- and bone marrow-derived progenitors. *Haematologica*.

Baddoo M, Hill K, Wilkinson R, Gaupp D, Hughes C, Kopen GC, Phinney DG. 2003. Characterization of mesenchymal stem cells isolated from murine bone marrow by negative selection. *J Cell Biochem*.

Baker M. 2016. Reproducibility: Respect your cells! *Nature*.

Ball LM, Bernardo ME, Roelofs H, Lankester A, Cometa AM, Egeler RM, Locatelli F, Fibbe WE. 2007. Cotransplantation of ex vivo-expanded mesenchymal stem cells accelerates

lymphocyte recovery and may reduce the risk of graft failure in haploidentical hematopoietic stem-cell transplantation. *Blood* **110**: 2764–2767.

Banfi A, Muraglia A, Dozin B, Mastrogiacomo M, Cancedda R, Quarto R. 2000. Proliferation kinetics and differentiation potential of ex vivo expanded human bone marrow stromal cells: Implications for their use in cell therapy. *Exp Hematol* **28**: 707–715.

Barkholt L, Flory E, Jekerle V, Lucas-Samuel S, Ahnert P, Bisset L, Büscher D, Fibbe WE, Foussat A, Kwa M, et al. 2013. Risk of tumorigenicity in mesenchymal stromal cell-based therapies - Bridging scientific observations and regulatory viewpoints. *Cytotherapy* **15**: 753–759.

Bartmann C, Rohde E, Schallmoser K, Pürstner P, Lanzer G, Linkesch W, Strunk D. 2007. Two steps to functional mesenchymal stromal cells for clinical application. *Transfusion* **47**: 1426–1435.

Bartunek J, Behfar A, Dolatabadi D, Vanderheyden M, Ostojic M, Dens J, El Nakadi B, Banovic M, Beleslin B, Vrolix M, et al. 2013. Cardiopoietic stem cell therapy in heart failure: The C-CURE (cardiopoietic stem cell therapy in heart failURE) multicenter randomized trial with lineage-specified biologics. *J Am Coll Cardiol* **61**: 2329–2338.

Batorov E V., Shevela EY, Tikhonova MA, Batorova DS, Ushakova GY, Sizikova SA, Sergeevicheva V V., Gilevich A V., Kryuchkova I V., Ostanin AA, et al. 2015. Mesenchymal stromal cells improve early lymphocyte recovery and T cell reconstitution after autologous hematopoietic stem cell transplantation in patients with malignant lymphomas. *Cell Immunol* **297**: 80–86.

Baxter MA, Wynn RF, N. JS, Wraith JE, Fairbairn LJ, Bellatuono I. 2004. Study of Telomere Length Reveals Rapid Aging of Human Marrow Stromal Cells following In Vitro Expansion. *Stem Cells* **22**: 675–682.

Becherucci V, Piccini L, Casamassima S, Bisin S, Gori V, Gentile F, Ceccantini R, De Rienzo E, Bindi B, Pavan P, et al. 2018. Human platelet lysate in mesenchymal stromal cell expansion according to a GMP grade protocol: A cell factory experience. *Stem Cell Res Ther* **9**.

Ben-Porath I, Weinberg RA. 2005. The signals and pathways activating cellular senescence. *Int J Biochem Cell Biol* **37**: 961–76.

Benvenuto F, Ferrari S, Gerdoni E, Gualandi F, Frassoni F, Pistoia V, Mancardi G, Uccelli A. 2007. Human Mesenchymal Stem Cells Promote Survival of T Cells in a Quiescent State. *Stem Cells* **25**: 1753–1760.

Bernal A, Arranz L. 2018. Nestin-expressing progenitor cells: function, identity and therapeutic implications. *Cell Mol Life Sci* **75**: 2177–2195. https://doi.org/10.1007/s00018-018-2794-z.

Bernardo ME, Avanzini MA, Perotti C, Cometa AM, Moretta A, Lenta E, Del Fante C, Novara F, De Silvestri A, Amendola G, et al. 2007a. Optimization of in vitro expansion of human multipotent mesenchymal stromal cells for cell-therapy approaches: Further insights in the search for a fatal calf serum substitute. *J Cell Physiol* **211**: 121–30.

Bernardo ME, Avanzini MA, Perotti C, Cometa AM, Moretta A, Lenta E, Del Fante C, Novara F, Zuffardi O, Maccario R, et al. 2006. Platelet-Lysate for In Vitro Expansion of Human Multipotent Mesenchymal Stromal Cells in Approaches of Cell-Therapy. *Blood*.

Bernardo ME, Ball LM, Cometa AM, Roelofs H, Zecca M, Avanzini MA, Bertaina A, Vinti L, Lankester A, MacCario R, et al. 2011. Co-infusion of ex vivo-expanded, parental MSCs prevents life-threatening acute GVHD, but does not reduce the risk of graft failure in pediatric patients undergoing allogeneic umbilical cord blood transplantation. *Bone Marrow Transplant* **46**: 200–207.

Bernardo ME, Emons JAM, Karperien M, Nauta AJ, Willemze R, Roelofs H, Romeo S, Marchini A, Rappold GA, Vukicevic S, et al. 2007b. Human mesenchymal stem cells derived from bone marrow display a better chondrogenic differentiation compared with other sources. *Connect Tissue Res*.

Bertolo A, Baur M, Guerrero J, Pötzel T, Stoyanov J. 2019. Autofluorescence is a Reliable in vitro Marker of Cellular Senescence in Human Mesenchymal Stromal Cells. *Sci Rep* **9**.

Bertolo A, Mehr M, Janner-Jametti T, Graumann U, Aebli N, Baur M, Ferguson SJ, Stoyanov J V. 2016. An in vitro expansion score for tissue-engineering applications with human bone marrow-derived mesenchymal stem cells. *J Tissue Eng Regen Med* **10**: 149–161.

Beyth S, Borovsky Z, Mevorach D, Liebergall M, Gazit Z, Aslan H, Galun E, Rachmilewitz J. 2005. Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness. *Blood* **105**: 2214–9.

Bianco P, Cao X, Frenette PS, Mao JJ, Robey PG, Simmons PJ, Wang CY. 2013. The meaning, the sense and the significance: Translating the science of mesenchymal stem cells into medicine. *Nat Med* **19**: 35–42.

Bieback K, Brinkmann I. 2010. Mesenchymal stromal cells from human perinatal tissues: From biology to cell therapy. *World J Stem Cells* **2**: 81.

Bieback K, Hecker A, Kocaömer A, Lannert H, Schallmoser K, Strunk D, Klüter H. 2009. Human alternatives to fetal bovine serum for the expansion of mesenchymal stromal cells from bone marrow. *Stem Cells* **27**: 2331–2341.

Bieback K, Hecker A, Schlechter T, Hofmann I, Brousos N, Redmer T, Besser D, Klter H, Mller AM, Becker M. 2012. Replicative aging and differentiation potential of human adipose tissue-derived mesenchymal stromal cells expanded in pooled human or fetal bovine serum. *Cytotherapy* **14**: 570–583.

Bieback K, Kern S, Kocaömer A, Ferlik K, Bugert P. 2008. Comparing mesenchymal stromal cells from different human tissues: Bone marrow, adipose tissue and umbilical cord blood. *Biomed Mater Eng* **18**: 71–76.

Bieback K, Kinzebach S, Karagianni M. 2011. Translating research into clinical scale manufacturing of mesenchymal stromal cells. *Stem Cells Int* **2010**.

Bieback K, Kuçi S, Schäfer R. 2019. Production and quality testing of multipotent mesenchymal stromal cell therapeutics for clinical use. *Transfusion* **59**: 2164–2173.

Bieback K, Netsch P. 2016. Isolation, culture, and characterization of human umbilical cord blood-derived mesenchymal stromal cells. In *Methods in Molecular Biology*.

Bigot N, Mouche A, Preti M, Loisel S, Renoud ML, Le Guével R, Sensebé L, Tarte K, Pedeux R. 2015. Hypoxia Differentially Modulates the Genomic Stability of Clinical-Grade ADSCs and BM-MSCs in Long-Term Culture. *Stem Cells* **33**: 3608–3620.

Birmingham E, Niebur GL, Mchugh PE, Shaw G, Barry FP, McNamara LM. 2012. Osteogenic differentiation of mesenchymal stem cells is regulated by osteocyte and osteoblast cells in a simplified bone niche. *Eur Cells Mater* **23**: 13–27.

Bonab MM, Sahraian MA, Aghsaie A, Karvigh SA, Hosseinian SM, Nikbin B, Lotfi J, Khorramnia S, Motamed MR, Togha M, et al. 2012. Current Stem Cell Autologous Mesenchymal Stem Cell Therapy in Progressive Multiple Sclerosis: An Open Label Study. *Res Ther* **7**: 407–414.

Bork S, Pfister S, Witt H, Horn P, Korn B, Ho AD, Wagner W. 2010. DNA methylation pattern changes upon long-term culture and aging of human mesenchymal stromal cells. *Aging Cell* **9**: 54–63.

Bosnakovski D, Mizuno M, Kim G, Takagi S, Okumura M, Fujinaga T. 2005. Isolation and multilineage differentiation of bovine bone marrow mesenchymal stem cells. *Cell Tissue Res* **319**: 243–53.

Bruder SP, Jaiswal N, Haynesworth SE. 1997. Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. *J Cell Biochem* **64**: 278–294.

Brunk UT, Terman A. 2002. Lipofuscin: Mechanisms of age-related accumulation and influence on cell function. *Free Radic Biol Med* **33**: 611–619.

Burand AJ, Gramlich OW, Brown AJ, Ankrum JA. 2017. Function of Cryopreserved Mesenchymal Stromal Cells With and Without Interferon-γ Prelicensing is Context Dependent. *Stem Cells* **35**: 1437–1439.

Burnouf T, Goubran HA, Chen TM, Ou KL, El-Ekiaby M, Radosevic M. 2013. Blood-derived biomaterials and platelet growth factors in regenerative medicine. *Blood Rev* **27**: 77–89.

Burova E, Borodkina A, Shatrova A, Nikolsky N. 2013. Sublethal oxidative stress induces the premature senescence of human mesenchymal stem cells derived from endometrium. *Oxid Med Cell Longev*.

Cai J, Wu Z, Xu X, Liao L, Chen J, Huang L, Wu W, Luo F, Wu C, Pugliese A, et al. 2016. Umbilical Cord Mesenchymal Stromal Cell with Autologous Bone Marrow Cell Transplantation in Established Type 1 Diabetes: A Pilot Randomized Controlled Open-

Label Clinical Study to Assess Safety and Impact on Insulin Secretion. *Diabetes Care* **39**: 149–157.
Cao Y, Ding Z, Han C, Shi H, Cui L, Lin R. 2017. Efficacy of Mesenchymal Stromal Cells for Fistula Treatment of Crohn's Disease: A Systematic Review and Meta-Analysis. *Dig Dis Sci* **62**: 851–860.
Capelli C, Domenghini M, Borleri G, Bellavita P, Poma R, Carobbio A, Micò C, Rambaldi A, Golay J, Introna M. 2007. Human platelet lysate allows expansion and clinical grade production of mesenchymal stromal cells from small samples of bone marrow aspirates or marrow filter washouts. *Bone Marrow Transplant* **40**: 785–91.
Caplan AI. 2008. All MSCs Are Pericytes? *Cell Stem Cell* **3**: 229–230.
Caplan AI. 1991. Mesenchymal stem cells. *J Orthop Res* **9**: 641-650J.
Caplan AI. 2017. New MSC: MSCs as pericytes are Sentinels and gatekeepers. *J Orthop Res* **35**: 1151–1159.
Carlsson PO, Schwarcz E, Korsgren O, Le Blanc K. 2015. Preserved β-cell function in type 1 diabetes by mesenchymal stromal cells. *Diabetes* **64**: 587–592.
Castello LM, Leone M, Adamini A, Castiglia S, Mareschi K, Ferrero I, De Gobbi Marco †, Carnevale-Schianca F, Fagioli F, Berger M. 2018. *Analysis of Mesenchymal Stromal Cell Engraftment After Allogeneic HSCT in Pediatric Patients: A Large Multicenter Study*.
Castrén E, Sillat T, Oja S, Noro A, Laitinen A, Konttinen YT, Lehenkari P, Hukkanen M, Korhonen M. 2015. Osteogenic differentiation of mesenchymal stromal cells in two-dimensional and three-dimensional cultures without animal serum. *Stem Cell Res Ther* **6**.
Castro-Malaspina H, Gay R., Resnick G, Kapoor N, Meyers P, D C, McKenzie S, Broxmeyer H, Moore MA. 1980. Characterization of human bone marrow fibroblast colony-forming cells (CFU-F) and their progeny. *Blood* **56**: 289–301.
Cervenakova L, Akimov S, Vasilyeva I, Yakovleva O, McKenzie C, Cervenak J, Piccardo P, Asher DM. 2011. Fukuoka-1 strain of transmissible spongiform encephalopathy agent infects murine bone marrow-derived cells with features of mesenchymal stem cells. *Transfusion* **51**: 1755–1768.
CFR - Code of Federal Regulations Title 21. 2013. Current Good Manufacturing Practice For Finished Pharmaceuticals. *Part 211 Curr Good Manuf Pract Finish Pharm*.
Chen CL, Mahjoubfar A, Tai LC, Blaby IK, Huang A, Niazi KR, Jalali B. 2016. Deep Learning in Label-free Cell Classification. *Sci Rep* **6**.
Chen L, Tredget EE, Wu PYG, Wu Y, Wu Y. 2008. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. *PLoS One*.
Chen X, Wang C, Yin J, Xu J, Wei J, Zhang Y, Del Cañizo MC. 2015. Efficacy of mesenchymal stem cell therapy for steroid-refractory acute graft-versus-host disease following allogeneic hematopoietic stem cell transplantation: A systematic review and meta-analysis. *PLoS One* **10**.
Chevallier N, Anagnostou F, Zilber S, Bodivit G, Maurin S, Barrault A, Bierling P, Hernigou P, Layrolle P, Rouard H. 2010. Osteoblastic differentiation of human mesenchymal stem cells with platelet lysate. *Biomaterials* **31**: 270–278.
Chiesa S, Morbelli S, Morando S, Massollo M, Marini C, Bertoni A, Frassoni F, Bartolomé ST, Sambuceti G, Traggiai E, et al. 2011. Mesenchymal stem cells impair in vivo T-cell priming by dendritic cells. *Proc Natl Acad Sci U S A* **108**: 17384–17389.
Childs BG, Durik M, Baker DJ, Van Deursen JM. 2015. Cellular senescence in aging and age-related disease: From mechanisms to therapy. *Nat Med* **21**: 1424–1435.
Chinnadurai R, Copland IB, Garcia MA, Petersen CT, Lewis CN, Waller EK, Kirk AD, Galipeau J. 2016. Cryopreserved Mesenchymal Stromal Cells Are Susceptible to T-Cell Mediated Apoptosis Which Is Partly Rescued by IFNγ Licensing. *Stem Cells* **34**: 2429–2442.
Chinnadurai R, Copland IB, Patel SR, Galipeau J. 2014a. IDO-Independent Suppression of T Cell Effector Function by IFN-γ–Licensed Human Mesenchymal Stromal Cells. *J Immunol* **192**: 1491–1501.
Chinnadurai R, Garcia MA, Sakurai Y, Lam WA, Kirk AD, Galipeau J, Copland IB. 2014b. Actin cytoskeletal disruption following cryopreservation alters the biodistribution of human mesenchymal stromal cells in vivo. *Stem Cell Reports* **3**: 60–72.
Chinnadurai R, Rajan D, Ng S, McCullough K, Arafat D, Waller EK, Anderson LJ, Gibson G,

Galipeau J. 2017. Immune dysfunctionality of replicative senescent mesenchymal stromal cells is corrected by IFNg priming. *Blood Adv* **1**: 628–643.
Cho KA, Sung JR, Yoon SO, Ji HP, Jung WL, Kim HP, Kyung TK, Ik SJ, Sang CP. 2004. Morphological adjustment of senescent cells by modulating caveolin-1 status. *J Biol Chem* **279**: 42270–42278.
Chow DC, Wenning LA, Miller WM, Papoutsakis ET. 2001. Modeling pO 2 Distributions in the Bone Marrow Hematopoietic Compartment. I. Krogh's Model. **81**: 675–84.
Chullikana A, Majumdar A Sen, Gottipamula S, Krishnamurthy S, Kumar AS, Prakash VS, Gupta PK. 2015. Randomized, double-blind, phase I/II study of intravenous allogeneic mesenchymal stromal cells in acute myocardial infarction. *Cytotherapy* **17**: 250–261.
Cohneim JF. 1867. Ueber Entzündung und Eiterung. *Arch Pathol Anat Pysiol Klin Med* **40**: 1–79.
Colter DC, Sekiya I, Prockop DJ. 2010. Identification of a subpopulation of rapidly and renewing multipotential in colonies of human marrow stromal cells. *Proc Natl Acad Sci U S A* **98**: 7841–7845.
Connick P. 2012. Autologous mesenchymal stem cells for the treatment of secondary progressive multiple sclerosis: an open-label phase 2a proof-of-concept study. *Artic Lancet Neurol* **11**: 150–56.
Coppé J-P, Desprez P-Y, Krtolica A, Campisi J. 2010. The Senescence-Associated Secretory Phenotype: The Dark Side of Tumor Suppression. *Annu Rev Pathol Mech Dis* **5**: 99–118.
Coppé J-P, Patil CK, Rodier F, Sun Y, Muñoz DP, Goldstein J, Nelson PS, Desprez PY, Campisi J. 2008. Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. *PLoS Biol* **6**: 2852–68.
Corcione A, Benvenuto F, Ferretti E, Giunti D, Cappiello V, Cazzanti F, Risso M, Gualandi F, Mancardi GL, Pistoia V, et al. 2006. Human mesenchymal stem cells modulate B-cell functions. *Blood* **107**: 367–372.
Crippa S, Bernardo ME. 2018. Mesenchymal Stromal Cells: Role in the BM Niche and in the Support of Hematopoietic Stem Cell Transplantation. *HemaSphere* **2**: e151.
Crisan M, Corselli M, Chen WCW, Péault B. 2012. Perivascular cells for regenerative medicine. *J Cell Mol Med* **16**: 2851–2860.
Crisan M, Yap S, Casteilla L, Chen CW, Corselli M, Park TS, Andriolo G, Sun B, Zheng B, Zhang L, et al. 2008. A Perivascular Origin for Mesenchymal Stem Cells in Multiple Human Organs. *Cell Stem Cell*.
Cristofalo VJ, Allen RG, Pignolo RJ, Martin BG, Beck JC. 1998. Relationship between donor age and the replicative lifespan of human cells in culture: A reevaluation. *Proc Natl Acad Sci U S A*.
da Silva Meirelles L, Caplan AI, Nardi NB. 2008. In Search of the In Vivo Identity of Mesenchymal Stem Cells. *Stem Cells* **26**: 2287–2299.
da Silva Meirelles L, Chagastelles PC, Nardi NB. 2006. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *J Cell Sci* **119**: 2204–2213.
Davalos AR, Coppé J-P, Campisi J, Desprez PY. 2010. Senescent cells as a source of inflammatory factors for tumor progression. *Cancer Metastasis Rev* **29**: 273–283.
Dazzi F, Ramasamy R, Glennie S, Jones SP, Roberts I. 2006. The role of mesenchymal stem cells in haemopoiesis. *Blood Rev* **20**: 161–171.
De Almeida DC, Ferreira MRP, Franzen J, Weidner CI, Frobel J, Zenke M, Costa IG, Wagner W. 2016. Epigenetic Classification of Human Mesenchymal Stromal Cells. *Stem Cell Reports* **6**: 168–175. http://dx.doi.org/10.1016/j.stemcr.2016.01.003.
De Bari C, Dell'Accio F, Tylzanowski P, Luyten FP. 2001. Multipotent mesenchymal stem cells from adult human synovial membrane. *Arthritis Rheum*.
de Lange T. 2009. How Telomeres Solve the End-Protection Problem. *Science (80-)* **326**: 948.
de Lange T. 2005. Shelterin: The protein complex that shapes and safeguards human telomeres. *Genes Dev* **19**: 2100–10.
De Witte S, Lambert E, Merino A, Strini T, Douben H, O'Flynn L, Elliman SJ, de Klein A, Newsome P, Baan CC, et al. 2017. Aging of bone marrow– and umbilical cord–derived mesenchymal stromal cells during expansion. *Cytotherapy* **19**: 798–807.
Detry O, Vandermeulen M, Delbouille M-H, Somja J, Bletard N, Briquet A, Lechanteur C, Giet

O, Baudoux E, Hannon M, et al. 2017. Infusion of mesenchymal stromal cells after deceased liver transplantation: A phase I-II, open-label, clinical study. *J Hepatol* **67**: 47–55. www.misot.eu.

Devine SM, Bartholomew AM, Mahmud N, Nelson M, Patil S, Hardy W, Sturgeon C, Hewett T, Chung T, Stock W, et al. 2001. Mesenchymal stem cells are capable of homing to the bone marrow of non-human primates following systemic infusion. *Exp Hematol*.

Dezawa M, Ishikawa H, Itokazu Y, Yoshihara T, Hoshino M, Takeda SI, Ide C, Nabeshima YI. 2005. Developmental biology: Bone marrow stromal cells generate muscle cells and repair muscle degeneration. *Science (80-)* **309**: 314–317.

Dhere T, Copland IB, Garcia M, Chiang KY, Chinnadurai R, Prasad M, Galipeau J, Kugathasan S. 2016. The safety of autologous and metabolically fit bone marrow mesenchymal stromal cells in medically refractory Crohn's disease – a phase 1 trial with three doses. *Aliment Pharmacol Ther* **44**: 471–481.

di Fagagna A, Reaper PM, Clay-Farrace L, Fiegler H, Carr P, von Zglinicki T, Saretzki G, Carter NP, Jackson SP. 2003. A DNA damage checkpoint response in telomere-initiated senescence. *Nature* **426**: 194–198.

Di GH, Liu Y, Lu Y, Liu J, Wu C, Duan HF. 2014. IL-6 secreted from senescent mesenchymal stem cells promotes proliferation and migration of breast cancer cells. *PLoS One* **9**.

Di Nicola M, Carlo-Stella C, Magni M, Milanesi M, Longoni PD, Matteucci P, Grisanti S, Gianni AM. 2002. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* **99**: 3838–43.

Digirolamo CM, Stokes D, Colter DC, Phinney DG, Class R, Prockop DJ. 1999. Propagation and senescence of human marrow stromal cells in culture: A simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. *Br J Haematol* **107**: 275–281.

Dimri GP, Itahana K, Acosta M, Campisi J. 2000. Regulation of a Senescence Checkpoint Response by the E2F1 Transcription Factor and p14ARF Tumor Suppressor. *Mol Cell Biol* **20**: 273–285.

Dimri GP, Leet X, Basile G, Acosta M, Scorrt G, Roskelley C, Medrano EE, Linskensi M, Rubeljii I, Pereira-Smithii O, et al. 1995. A biomarker that identifies senescent human cells in culture and in aging skin in vivo (replicative senescence/tumor suppression/18-galactosidase) Communicated by Arthur. *Cell Bioiogy* **92**: 9363–9367.

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini FC, Krause DS, Deans RJ, Keating A, Prockop DJ, Horwitz EM. 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*.

Dormont D. 1999. Transmissible spongiform encephalopathy agents and animal sera. *Dev Biol Stand*.

Dotoli GM, De Santis GC, Orellana MD, De Lima Prata K, Caruso SR, Fernandes TR, Rensi Colturato VA, Kondo AT, Hamerschlak N, Simões BP, et al. 2017. Mesenchymal stromal cell infusion to treat steroid-refractory acute GvHD III/IV after hematopoietic stem cell transplantation. *Bone Marrow Transplant* **52**: 859–862.

Doucet C, Ernou I, Zhang Y, Llense JR, Begot L, Holy X, Lataillade JJ. 2005. Platelet lysates promote mesenchymal stem cell expansion: A safety substitute for animal serum in cell-based therapy applications. *J Cell Physiol* **205**: 228–236.

Du Rocher B, Mencalha AL, Gomes BE, Abdelhay E. 2012. Mesenchymal stromal cells impair the differentiation of CD14++ CD16- CD64+ classical monocytes into CD14++ CD16+ CD64++ activate monocytes. *Cytotherapy* **14**: 12–25.

Dubois B, Bridon JM, Fayette J, Barthélémy C, Banchereau J, Caux C, Brière F. 1999. Dendritic cells directly modulate B cell growth and differentiation. *J Leukoc Biol*.

Duijvestein M, Vos ACW, Roelofs H, Wildenberg ME, Wendrich BB, Verspaget HW, Kooy-Winkelaar EMC, Koning F, Zwaginga JJ, Fidder HH, et al. 2010. Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: Results of a phase I study. *Gut* **59**: 1662–1669.

Dumont P, Burton M, Chen QM, Gonos ES, Frippiat C, Mazarati JB, Eliaers F, Remacle J, Toussaint O. 2000. Induction of replicative senescence biomarkers by sublethal oxidative

stresses in normal human fibroblast. *Free Radic Biol Med* **28**: 361–373.
Elahi KC, Klein G, Avci-Adali M, Sievert KD, Macneil S, Aicher WK. 2016. Human mesenchymal stromal cells from different sources diverge in their expression of cell surface proteins and display distinct differentiation patterns. *Stem Cells Int* **2016**.
Elsawa SF, Novak AJ, Ziesmer SC, Almada LL, Hodge LS, Grote DM, Witzig TE, Fernandez-Zapico ME, Ansell SM. 2011. Comprehensive analysis of tumor microenvironment cytokines in Waldenstrom macroglobulinemia identifies CCL5 as a novel modulator of IL-6 activity. *Blood* **118**: 5540–5549.
English K, Barry FP, Field-Corbett CP, Mahon BP. 2007. IFN-γ and TNF-α differentially regulate immunomodulation by murine mesenchymal stem cells. *Immunol Lett* **110**: 91–100.
Erices A, Conget P, Minguell JJ. 2000. Mesenchymal progenitor cells in human umbilical cord blood. *Br J Haematol*.
Estrada JC, Albo C, Benguría A, Dopazo A, López-Romero P, Carrera-Quintanar L, Roche E, Clemente EP, Enríquez JA, Bernad A, et al. 2012. Culture of human mesenchymal stem cells at low oxygen tension improves growth and genetic stability by activating glycolysis. *Cell Death Differ* **19**: 743–755.
Estrada JC, Torres Y, Benguría A, Dopazo A, Roche E, Carrera-Quintanar L, Pérez RA, Enríquez JA, Torres R, Ramírez JC, et al. 2013. Human mesenchymal stem cell-replicative senescence and oxidative stress are closely linked to aneuploidy. *Cell Death Dis* **4**.
European comission. 2017. EudraLex The Rules Governing Medicinal Products in the European Union: Guidelines on Good Manufacturing Practice specific to Advanced Therapy Medicinal Products. **4**.
Fadeel B, Åhlin A, Henter JI, Orrenius S, Hampton MB. 1998. Involvement of caspases in neutrophil apoptosis: Regulation by reactive oxygen species. *Blood* **92**: 4808–18.
Fehrer C, Brunauer R, Laschober G, Unterluggauer H, Reitinger S, Kloss F, Gülly C, Gaßner R, Lepperdinger G. 2007. Reduced oxygen tension attenuates differentiation capacity of human mesenchymal stem cells and prolongs their lifespan. *Aging Cell* **6**: 745–757.
Fekete N, Gadelorge M, Frst D, Maurer C, Dausend J, Fleury-Cappellesso S, Mailnder V, Lotfi R, Ignatius A, Sensebé L, et al. 2012. Platelet lysate from whole blood-derived pooled platelet concentrates and apheresis-derived platelet concentrates for the isolation and expansion of human bone marrow mesenchymal stromal cells: Production process, content and identification of active comp. *Cytotherapy* **14**: 540–554.
Fekete N, Rojewski M, Lotfi R, Schrezenmeier H. 2014. Essential components for Ex vivo proliferation of mesenchymal stromal cells. *Tissue Eng - Part C Methods* **20**: 129–139.
Fisher SA, Cutler A, Doree C, Brunskill SJ, Stanworth SJ, Navarrete C, Girdlestone J. 2019. Mesenchymal stromal cells as treatment or prophylaxis for acute or chronic graft-versus-host disease in haematopoietic stem cell transplant (HSCT) recipients with a haematological condition. *Cochrane Database Syst Rev* **2019**.
Fitzsimmons REB, Mazurek MS, Soos A, Simmons CA. 2018. Mesenchymal stromal/stem cells in regenerative medicine and tissue engineering. *Stem Cells Int* **2018**.
Forbes GM, Sturm MJ, Leong RW, Sparrow MP, Segarajasingam D, Cummins AG, Phillips M, Herrmann RP. 2014. A phase 2 study of allogeneic mesenchymal stromal cells for luminal crohn's disease refractory to biologic therapy. *Clin Gastroenterol Hepatol* **12**: 64–71.
François M, Copland IB, Yuan S, Romieu-Mourez R, Waller EK, Galipeau J. 2012a. Cryopreserved mesenchymal stromal cells display impaired immunosuppressive properties as a result of heat-shock response and impaired interferon-γ licensing. *Cytotherapy* **14**: 147–152.
François M, Romieu-Mourez R, Li M, Galipeau J. 2012b. Human MSC suppression correlates with cytokine induction of indoleamine 2,3-dioxygenase and bystander M2 macrophage differentiation. *Mol Ther* **20**: 187–195.
Franzen J, Wagner W, Fernandez-Rebollo E. 2016. Epigenetic Modifications upon Senescence of Mesenchymal Stem Cells. *Curr Stem Cell Reports* **16**: 183–191.
Friedenstein A. 1976. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp Hematol* **4**: 274.
Fu W-Y, Lu Y-M, Piao Y-J. 2001. Differentiation and telomerase activity of human mesenchymal stem cells. **21**: 801–805.

Galipeau J. 2020. Mesenchymal Stromal Cells for Graft-versus-Host Disease: A Trilogy. *Biol Blood Marrow Transplant* **26**: e89–e91. https://doi.org/10.1016/j.bbmt.2020.02.023.
Galipeau J. 2017. Reply: "Function of Cryopreserved Mesenchymal Stromal Cells With and Without Interferon-γ Prelicensing Is Context Dependent." *Stem Cells* **35**: 1440–1441.
Galipeau J. 2013. The mesenchymal stromal cells dilemma-does a negative phase III trial of random donor mesenchymal stromal cells in steroid-resistant graft-versus-host disease represent a death knell or a bump in the road? *Cytotherapy* **15**: 2–8.
Galipeau J, Krampera M, Barrett J, Dazzi F, Deans RJ, DeBruijn J, Dominici M, Fibbe WE, Gee AP, Gimble JM, et al. 2015. International Society for Cellular Therapy perspective on immune functional assays for mesenchymal stromal cells as potency release criterion for advanced phase clinical trials. *Cytotherapy* **18**: 151–159.
Galipeau J, Sensebé L. 2018. Mesenchymal Stromal Cells: Clinical Challenges and Therapeutic Opportunities. *Cell Stem Cell* **22**: 824–833.
Galleu A, Riffo-Vasquez Y, Trento C, Lomas C, Dolcetti L, Cheung TS, Von Bonin M, Barbieri L, Halai K, Ward S, et al. 2017. Apoptosis in mesenchymal stromal cells induces in vivo recipient-mediated immunomodulation. *Sci Transl Med* **9**: 1–11.
Gao S, Mao F, Zhang B, Zhang L, Zhang X, Wang M, Yan Y, Yang T, Zhang J, Zhu W, et al. 2014. Mouse bone marrow-derived mesenchymal stem cells induce macrophage M2 polarization through the nuclear factor-κB and signal transducer and activator of transcription 3 pathways. *Exp Biol Med* **239**: 366–375.
Garcia-Olmo D, Herreros D, Pascual I, Pascual JA, Del-Valle E, Zorrilla J, De-La-Quintana P, Garcia-Arranz M, Pascual M. 2009. Expanded adipose-derived stem cells for the treatment of complex perianal fistula: A phase ii clinical trial. *Dis Colon Rectum* **52**: 79–86.
Geißler S, Textor M, Kühnisch J, Könnig D, Klein O, Ode A, Pfitzner T, Adjaye J, Kasper G, Duda GN. 2012. Functional Comparison of Chronological and In Vitro Aging: Differential Role of the Cytoskeleton and Mitochondria in Mesenchymal Stromal Cells. *PLoS One* **7**.
Gerosa F, Baldani-Guerra B, Nisii C, Marchesini V, Carra G, Trinchieri G. 2002. Reciprocal activating interaction between natural killer cells and dendritic cells. *J Exp Med* **195**: 327–33.
Ghannam S, Pène J, Torcy-Moquet G, Jorgensen C, Yssel H. 2010. Mesenchymal Stem Cells Inhibit Human Th17 Cell Differentiation and Function and Induce a T Regulatory Cell Phenotype. *J Immunol* **185**: 302–312.
Gimbel M, Ashley RK, Sisodia M, Gabbay JS, Wasson KL, Heller J, Wilson L, Kawamoto HK, Bradley JP. 2007. Repair of alveolar cleft defects: Reduced morbidity with bone marrow stem cells in a resorbable matrix. *J Craniofac Surg* **18**: 895–901.
Glenn JD, Whatenby K. 2014. Mesenchymal stem cells: Emerging mechanisms of immunomodulation and therapy. *World J Stem Cells* **6**: 526.
Glennie S, Soeiro I, Dyson PJ, Lam EWF, Dazzi F. 2005. Bone marrow mesenchymal stem cells induce division arrest anergy of activated T cells. *Blood* **105**: 2821–7.
Gosselin K, Deruy E, Martien S, Vercamer C, Bouali F, Dujardin T, Slomianny C, Houel-Renault L, Chelli F, De Launoit Y, et al. 2009. Senescent keratinocytes die by autophagic programmed cell death. *Am J Pathol* **174**: 423–435.
Goujon E. 1869. Recersches expérimentales sur les propriétés physiologiques de la moelle des os. *J Anat Physiol* **6**: 399–412.
Grau-Vorster M, Laitinen A, Nystedt J, Vives J. 2019. HLA-DR expression in clinical-grade bone marrow-derived multipotent mesenchymal stromal cells: A two-site study. *Stem Cell Res Ther* **10**.
Grayson WL, Zhao F, Bunnell B, Ma T. 2007. Hypoxia enhances proliferation and tissue formation of human mesenchymal stem cells. *Biochem Biophys Res Commun* **358**: 948–953.
Grayson WL, Zhao F, Izadpanah R, Bunnell B, Ma T. 2006. Effects of hypoxia on human mesenchymal stem cell expansion and plasticity in 3D constructs. *J Cell Physiol* **207**: 331–339.
Grégoire C, Ritacco C, Hannon M, Seidel L, Delens L, Belle L, Dubois S, Vériter S, Lechanteur C, Briquet A, et al. 2019. Comparison of mesenchymal stromal cells from different origins for the treatment of graft-vs.-host-disease in a humanized mouse model. *Front Immunol* **10**:

1–16.
Gregory CA, Singh H, Perry AS, Prockop DJ. 2003. The Wnt signaling inhibitor Dickkopf-1 is required for reentry into the cell cycle of human adult stem cells from bone marrow. *J Biol Chem* **278**: 28067–28078.
Griffiths S, Baraniak PR, Copland IB, Nerem RM, McDevitt TC. 2013. Human platelet lysate stimulates high-passage and senescent human multipotent mesenchymal stromal cell growth and rejuvenation in vitro. *Cytotherapy* **15**: 1469–1483.
Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. 2000. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proc Natl Acad Sci U S A*.
Gstraunthaler G, Lindl T, Van Der Valk J. 2013. A plea to reduce or replace fetal bovine serum in cell culture media. *Cytotechnology* **65**: 791–3.
Haack-Sørensen M, Juhl M, Follin B, Harary Søndergaard R, Kirchhoff M, Kastrup J, Ekblond A. 2018. Development of large-scale manufacturing of adipose-derived stromal cells for clinical applications using bioreactors and human platelet lysate. *Scand J Clin Lab Invest* **78**: 293–300. https://doi.org/10.1080/00365513.2018.1462082.
Hara E, Uzman JA, Dimri GP, Nehlin JO, Testori A, Campisi J. 1996. The helix-loop-helix protein Id-1 and a retinoblastoma protein binding mutant of SV40 T antigen synergize to reactivate DNA synthesis in senescent human fibroblasts. *Dev Genet* **18**: 161–172.
Hare JM, Fishman JE, Gerstenblith G, DiFede Velazquez DL, Zambrano JP, Suncion VY, Tracy M, Ghersin E, Johnston P V., Brinker JA, et al. 2012. Comparison of allogeneic vs autologous bone marrow-derived mesenchymal stem cells delivered by transendocardial injection in patients with ischemic cardiomyopathy: The POSEIDON randomized trial. *JAMA - J Am Med Assoc* **308**: 2369–2379.
Harley CB, Futcher B, Greider CW. 1990. Telomeres shorten during ageing of human fibroblasts. *Nature*.
Hayes M, Curley G, Ansari B, Laffey JG. 2012. Clinical review: Stem cell therapies for acute lung injury/acute respiratory distress syndrome - hope or hype? *Crit Care* **16**: 205.
Hayflick L. 1965. The limited in vitro lifetime of human diploid cell strains. *Exp Cell Res* **37**: 614–36.
Hayflick L, Moorhead PS. 1961. The serial cultivation of human diploid cell strains. *Exp Cell Res* **25**: 585–621.
Heiskanen A, Satomaa T, Tiitinen S, Laitinen A, Mannelin S, Impola U, Mikkola M, Olsson C, Miller-Podraza H, Blomqvist M, et al. 2007. N -Glycolylneuraminic Acid Xenoantigen Contamination of Human Embryonic and Mesenchymal Stem Cells Is Substantially Reversible . *Stem Cells* **25**: 197–202.
Heo JS, Choi Y, Kim HS, Kim HO. 2016. Comparison of molecular profiles of human mesenchymal stem cells derived from bone marrow, umbilical cord blood, placenta and adipose tissue. *Int J Mol Med* **37**: 115–125.
Ho AD, Wagner W, Franke W. 2008. Heterogeneity of mesenchymal stromal cell preparations. *Cytotherapy* **10**: 320–330.
Honda S, Weigel A, Hjelmeland LM, Handa JT. 2001. Induction of telomere shortening and replicative senescence by cryopreservation. *Biochem Biophys Res Commun*.
Hoogduijn MJ, De Witte SFH, Luk F, Van Den Hout-Van Vroonhoven MCGN, Ignatowicz L, Catar R, Strini T, Korevaar SS, Van Ijcken WFJ, Betjes MGH, et al. 2016. Effects of Freeze-Thawing and Intravenous Infusion on Mesenchymal Stromal Cell Gene Expression. *Stem Cells Dev* **25**: 586–597.
Horwitz EM. 2004. Dkk-1-mediated expansion of adult stem cells. *Trends Biotechnol* **22**: 386–388.
Horwitz EM, Le Blanc K, Dominici M, Mueller I, Slaper-Cortenbach I, Marini FC, Deans RJ, Krause DS, Keating A. 2005. Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. *Cytotherapy* **7**: 393–395.
Hunt CJ. 2011. Cryopreservation of human stem cells for clinical application: A review. *Transfus Med Hemotherapy* **38**: 107–123.
In 't Anker P, Scherjon S, Kleijburg-van der Keur C, de Groot-Swings G, Claas FHJ, Fibbe WE, Kanhai H. 2004. Isolation of Mesenchymal Stem Cells of Fetal or Maternal Origin from Human Placenta. *Stem Cells* **22**: 1338–45.

In 't Anker P, Scherjon S, Kleijburg-van der Keur C, Noort WA, Claas FHJ, Willemze R, Fibbe WE, Kanhai H. 2003. Amniotic fluid as a novel source of mesenchymal stem cells for therapeutic transplantation. *Blood* **102**: 1548–9.

Itahana K, Dimri GP, Campisi J. 2001. Regulation of cellular senescence by p53. *Eur J Biochem*.

Ito A, Aoyama T, Yoshizawa M, Nagai M, Tajino J, Yamaguchi S, Iijima H, Zhang X, Kuroki H. 2015. The effects of short-term hypoxia on human mesenchymal stem cell proliferation, viability and p16 INK4A mRNA expression: Investigation using a simple hypoxic culture system with a deoxidizing agent. *J Stem Cells Regenetrative Med* **11**: 25–31.

Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP. 1997. Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. *J Cell Biochem* **54**: 295–312.

Jeon ES, Moon HJ, Lee MJ, Song HY, Kim YM, Bae YC, Jung JS, Kim JH. 2006. Sphigosylphosphorylcholine induces differentiation of human mesenchymal stem cells into smooth-muscle-like through a TGF-β-dependent mechanism. *J Cell Sci* **119**: 4994–5005.

Jin HJ, Bae YK, Kim M, Kwon SJ, Jeon HB, Choi SJ, Kim SW, Yang YS, Oh W, Chang JW. 2013. Comparative analysis of human mesenchymal stem cells from bone marrow, adipose tissue, and umbilical cord blood as sources of cell therapy. *Int J Mol Sci* **14**: 17986–18001.

Jin Y, Kato T, Furu M, Nasu A, Kajita Y, Mitsui H, Ueda M, Aoyama T, Nakayama T, Nakamura T, et al. 2010. Mesenchymal stem cells cultured under hypoxia escape from senescence via down-regulation of p16 and extracellular signal regulated kinase. *Biochem Biophys Res Commun* **391**: 1471–1476.

Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU. 1998. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp Cell Res* **238**: 265–272.

Jonsdottir-Buch SM, Lieder R, Sigurjonsson OE. 2013. Platelet Lysates Produced from Expired Platelet Concentrates Support Growth and Osteogenic Differentiation of Mesenchymal Stem Cells. *PLoS One* **8**.

Kaigler D, Pagni G, Park CH, Braun TM, Holman LA, Yi E, Tarle SA, Bartel RL, Giannobile W V. 2013. Stem cell therapy for craniofacial bone regeneration: A randomized, controlled feasibility trial. *Cell Transplant* **22**: 767–777.

Kallekleiv M, Larun L, Bruserud Ø, Hatfield KJ. 2016. Co-transplantation of multipotent mesenchymal stromal cells in allogeneic hematopoietic stem cell transplantation: A systematic review and meta-analysis. *Cytotherapy* **18**: 172–185.

Karnoub AE, Dash AB, Vo AP, Sullivan A, Brooks MW, Bell GW, Richardson AL, Polyak K, Tubo R, Weinberg RA. 2007. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature* **449**: 557–563.

Karp JM, Leng Teo GS. 2009. Mesenchymal Stem Cell Homing: The Devil Is in the Details. *Cell Stem Cell* **4**: 206–16.

Karussis D, Karageorgiou C, Vaknin-Dembinsky A, Gowda-Kurkalli B, Gomori JM, Kassis I, Bulte JWM, Petrou P, Ben-Hur T, Abramsky O, et al. 2010. Safety and immunological effects of mesenchymal stem cell transplantation in patients with multiple sclerosis and amyotrophic lateral sclerosis. *Arch Neurol* **67**: 1187–1194.

Kasten P, Vogel J, Beyen I, Weiss S, Niemeyer P, Leo A, Luginbuhl R. 2008. Effect of platelet-rich plasma on the in vitro proliferation and osteogenic differentiation of human mesenchymal stem cells on distinct calcium phosphate scaffolds: The specific surface area makes a difference. *J Biomater Appl* **23**: 169–188.

Kastrup J, Haack-Sørensen M, Juhl M, Harary Søndergaard R, Follin B, Drozd Lund L, Mønsted Johansen E, Ali Qayyum A, Bruun Mathiasen A, Jørgensen E, et al. 2017. Cryopreserved Off-the-Shelf Allogeneic Adipose-Derived Stromal Cells for Therapy in Patients with Ischemic Heart Disease and Heart Failure—A Safety Study. *Stem Cells Transl Med* **6**: 1963–1971.

Katsube Y, Hirose M, Nakamura C, Ohgushi H. 2008. Correlation between proliferative activity and cellular thickness of human mesenchymal stem cells. *Biochem Biophys Res Commun* **368**: 256–260.

Kebriaei P, Hayes J, Daly A, Uberti J, Marks DI, Soiffer R, Waller EK, Burke E, Skerrett D, Shpall E, et al. 2019. A Phase 3 Randomized Study of Remestemcel-L versus Placebo Added to Second-Line Therapy in Patients with Steroid-Refractory Acute Graft-versus-Host

Disease. *Biol Blood Marrow Transplant*.
Kebriaei P, Isola L, Bahceci E, Holland K, Rowley S, McGuirk J, Devetten M, Jansen J, Herzig R, Schuster M, et al. 2009. Adult Human Mesenchymal Stem Cells Added to Corticosteroid Therapy for the Treatment of Acute Graft-versus-Host Disease. *Biol Blood Marrow Transplant* **15**: 804–811.
Kern S, Eichler H, Stoeve J, Klüter H, Bieback K. 2006. Comparative Analysis of Mesenchymal Stem Cells from Bone Marrow, Umbilical Cord Blood, or Adipose Tissue. *Stem Cells* **24**: 1294–1301.
Kharaziha P, Hellström PM, Noorinayer B, Farzaneh F, Aghajani K, Jafari F, Telkabadi M, Atashi A, Honardoost M, Zali MR, et al. 2009. Improvement of liver function in liver cirrhosis patients after autologous mesenchymal stem cell injection: A phase I-II clinical trial. *Eur J Gastroenterol Hepatol* **21**: 1199–1205.
Kim J, Hematti P. 2009. Mesenchymal stem cell-educated macrophages: A novel type of alternatively activated macrophages. *Exp Hematol* **37**: 1445–1453.
Kimura M, Stone RC, Hunt SC, Skurnick J, Lu X, Cao X, Harley CB, Aviv A. 2010. Measurement of telomere length by the southern blot analysis of terminal restriction fragment lengths. *Nat Protoc* **5**: 1596–1607.
Kirkwood TBL, Austad SN. 2000. Why do we age? *Nature*.
Klinker MW, Marklein RA, Lo Surdo JL, Wei CH, Bauer SR. 2017. Morphological features of IFN-γ-stimulated mesenchymal stromal cells predict overall immunosuppressive capacity. *Proc Natl Acad Sci U S A* **114**: E2598–E2607.
Koç ON, Gerson SL, Cooper BW, Dyhouse SM, Haynesworth SE, Caplan AI, Lazarus HM. 2000. Rapid hematopoietic recovery after coinfusion of autologous-blood stem cells and culture-expanded marrow mesenchymal stem cells in advanced breast cancer patients receiving high-dose chemotherapy. *J Clin Oncol*.
Kocaömer A, Kern S, Klüter H, Bieback K. 2007. Human AB Serum and Thrombin-Activated Platelet-Rich Plasma Are Suitable Alternatives to Fetal Calf Serum for the Expansion of Mesenchymal Stem Cells from Adipose Tissue. *Stem Cells* **25**: 1270–1278.
Koch CM, Joussen S, Schellenberg A, Lin Q, Zenke M, Wagner W. 2012. Monitoring of cellular senescence by DNA-methylation at specific CpG sites. *Aging Cell* **11**: 366–369.
Kozlowska U, Krawczenko A, Futoma K, Jurek T, Rorat M, Patrzalek D, Klimczak A. 2019. Similarities and differences between mesenchymal stem/progenitor cells derived from various human tissues. *World J Stem Cells* **11**: 347–374.
Krampera M, Cosmi L, Angeli R, Pasini A, Liotta F, Andreini A, Santarlasci V, Mazzinghi B, Pizzolo G, Vinante F, et al. 2006. Role for Interferon-γ in the Immunomodulatory Activity of Human Bone Marrow Mesenchymal Stem Cells. *Stem Cells* **24**: 386–398.
Krampera M, Glennie S, Dyson J, Scott D, Laylor R, Simpson E, Dazzi F. 2003. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. *Blood* **101**: 3722–3729.
Krampera M, Marconi S, Pasini A, Galiè M, Rigotti G, Mosna F, Tinelli M, Lovato L, Anghileri E, Andreini A, et al. 2007. Induction of neural-like differentiation in human mesenchymal stem cells derived from bone marrow, fat, spleen and thymus. *Bone* **40**: 382–390.
Krishna DR, Sperker B, Fritz P, Klotz U. 1999. Does pH 6 β-galactosidase activity indicate cell senescence? *Mech Ageing Dev* **109**: 113–123.
Krishnamurthy J, Torrice C, Ramsey MR, Sharpless NE, Kovalev GI, Su L, Al-Regaiey K. 2004. Ink4a/Arf expression is a biomarker of aging. *J Clin Invest* **114**: 1299–1307.
Kuilman T, Michaloglou C, Mooi WJ, Peeper DS. 2010. The essence of senescence. *Genes Dev* **24**: 2463–2479.
Kuilman T, Peeper DS. 2009. Senescence-messaging secretome: SMS-ing cellular stress. *Nat Rev Cancer*.
Kuo CK, Tuan RS. 2008. Mechanoactive tenogenic differentiation of human mesenchymal stem cells. *Tissue Eng - Part A* **14**: 1615–1627.
Kurtzberg J, Abdel-Azim H, Carpenter P, Chaudhury S, Horn B, Mahadeo K, Nemecek E, Neudorf S, Prasad V, Prockop S, et al. 2020. A Phase 3, Single-Arm, Prospective Study of Remestemcel-L, Ex Vivo Culture-Expanded Adult Human Mesenchymal Stromal Cells for the Treatment of Pediatric Patients Who Failed to Respond to Steroid Treatment for Acute

Graft-versus-Host Disease. *Biol Blood Marrow Transplant* **26**: 845–854. https://doi.org/10.1016/j.bbmt.2020.01.018.

Kurtzberg J, Prasad V, Grimley MS, Horn B, Carpenter PA, Jacobsohn D, Prockop S. 2010. Allogeneic Human Mesenchymal Stem Cell Therapy (Prochymal®) As A Rescue Agent For Severe Treatment Resistant GVHD In Pediatric Patients. *Biol Blood Marrow Transplant* **16**: S169. http://dx.doi.org/10.1016/j.bbmt.2009.12.056.

Kurtzberg J, Prockop S, Teira P, Bittencourt H, Lewis V, Chan KW, Horn B, Yu L, Talano JA, Nemecek E, et al. 2014. Allogeneic human mesenchymal stem cell therapy (Remestemcel-L, Prochymal) as a rescue agent for severe refractory acute graft-versus-host disease in pediatric patients. *Biol Blood Marrow Transplant* **20**: 229–235.

Kurz DJ, Decary S, Hong Y, Erusalimsky JD. 2000. Senescence-associated beta-galactosidase reflects an increase in lysosomal mass during replicative ageing of human endothelial cells. *J Cell Sci* **113**: 3613–22.

Kuznetsov SA, Mankani MH, Robey PG. 2000. Effect of serum on human bone marrow stromal cells: Ex vivo expansion and in vivo bone formation. *Transplantation* **70**: 1780–1787.

Laitinen A, Oja S, Kilpinen L, Kaartinen T, Möller J, Laitinen S, Korhonen M, Nystedt J. 2016a. A robust and reproducible animal serum-free culture method for clinical-grade bone marrow-derived mesenchymal stromal cells. *Cytotechnology* **68**: 891–906.

Laitinen A, Oja S, Kilpinen L, Kaartinen T, Möller J, Laitinen S, Korhonen M, Nystedt J. 2016b. A robust and reproducible animal serum-free culture method for clinical-grade bone marrow-derived mesenchymal stromal cells. *Cytotechnology* **68**.

Lalu MM, Mazzarello S, Zlepnig J, Dong YY (Ryan), Montroy J, McIntyre L, Devereaux PJ, Stewart DJ, David Mazer C, Barron CC, et al. 2018. Safety and Efficacy of Adult Stem Cell Therapy for Acute Myocardial Infarction and Ischemic Heart Failure (SafeCell Heart): A Systematic Review and Meta-Analysis. *Stem Cells Transl Med* **7**: 857–866.

Lalu MM, McIntyre L, Pugliese C, Fergusson D, Winston BW, Marshall JC, Granton J, Stewart DJ. 2012. Safety of Cell Therapy with Mesenchymal Stromal Cells (SafeCell): A Systematic Review and Meta-Analysis of Clinical Trials. *PLoS One* **7**.

Lange C, Cakiroglu F, Spiess AN, Cappallo-Obermann H, Dierlamm J, Zander AR. 2007. Accelerated and safe expansion of human mesenchymal stromal cells in animal serum-free medium for transplantation and regenerative medicine. *J Cell Physiol* **213**: 18–26.

Lazarus HM, Koc ON, Devine SM, Curtin P, Maziarz RT, Holland HK, Shpall EJ, McCarthy P, Atkinson K, Cooper BW, et al. 2005. Cotransplantation of HLA-identical sibling culture-expanded mesenchymal stem cells and hematopoietic stem cells in hematologic malignancy patients. *Biol Blood Marrow Transplant* **11**: 389–398.

Le Blanc K, Davies LC. 2015. Mesenchymal stromal cells and the innate immune response. *Immunol Lett* **168**: 140–146.

Le Blanc K, Frassoni F, Ball LM, Locatelli F, Roelofs H, Lewis I, Lanino E, Sundberg B, Bernardo ME, Remberger M, et al. 2008. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. *Lancet* **371**: 1579–1586.

Le Blanc K, Mougiakakos D. 2012. Multipotent mesenchymal stromal cells and the innate immune system. *Nat Rev Immunol* **12**: 383–396.

Le Blanc K, Rasmusson I, Sundberg B, Götherström C, Hassan M, Uzunel M, Ringdén O. 2004. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet* **363**: 1439–1441.

Lee JJ, Nam CE, Kook H, Maciejewski JP, Kim YK, Chung IJ, Park KS, Lee IK, Hwang TJ, Kim HJ. 2003. Constitution and telomere dynamics of bone marrow stromal cells in patients undergoing allogeneic bone marrow transplantation. *Bone Marrow Transplant* **32**: 947–952.

Lepperdinger G, Brunauer R, Jamnig A, Laschober G, Kassem M. 2008. Controversial issue: Is it safe to employ mesenchymal stem cells in cell-based therapies? *Exp Gerontol*.

Liang J, Zhang H, Hua B, Wang H, Lu L, Shi S, Hou Y, Zeng X, Gilkeson GS, Sun L. 2010. Allogenic mesenchymal stem cells transplantation in refractory systemic lupus erythematosus: A pilot clinical study. *Ann Rheum Dis* **69**: 1423–1429.

Liu D, O'Connor MS, Qin J, Songyang Z. 2004. Telosome, a mammalian telomere-associated complex formed by multiple telomeric proteins. *J Biol Chem* **279**: 51338–51342.

Lo Surdo JL, Millis BA, Bauer SR. 2013. Automated microscopy as a quantitative method to measure differences in adipogenic differentiation in preparations of human mesenchymal stromal cells. *Cytotherapy* **15**: 1527–1540.

Lohmann M, Walenda G, Hemeda H, Joussen S, Drescher W, Jockenhoevel S, Hutschenreuter G, Zenke M, Wagner W. 2012. Donor age of human platelet lysate affects proliferation and differentiation of mesenchymal stem cells. *PLoS One* **7**.

Lovelock JE, Bishop MWH. 1959. Prevention of freezing damage to living cells by dimethyl sulphoxide. *Nature* **183**: 1394–5.

Lucarelli E, Beccheroni A, Donati D, Sangiorgi L, Cenacchi A, Del Vento AM, Meotti C, Bertoja AZ, Giardino R, Fornasari PM, et al. 2003. Platelet-derived growth factors enhance proliferation of human stromal stem cells. *Biomaterials* **24**: 3095–3100.

Lucchini G, Introna M, Dander E, Rovelli A, Balduzzi A, Bonanomi S, Salvadè A, Capelli C, Belotti D, Gaipa G, et al. 2010. Platelet-lysate-expanded mesenchymal stromal cells as a salvage therapy for severe resistant graft-versus-host disease in a pediatric population. *Biol Blood Marrow Transplant* **16**: 1293–1301.

Lundberg AS, Weinberg RA. 1999. Control of the cell cycle and apoptosis. *Eur J Cancer* **35**: 1886–1894.

Lunyak V V., Amaro-Ortiz A, Gaur M. 2017. Mesenchymal stem cells secretory responses: Senescence messaging secretome and immunomodulation perspective. *Front Genet* **8**.

MacMillan ML, Blazar BR, DeFor TE, Wagner JE. 2009. Transplantation of ex-vivo culture-expanded parental haploidentical mesenchymal stem cells to promote engraftment in pediatric recipients of unrelated donor umbilical cord blood: Results of a phase I-II clinical trial. *Bone Marrow Transplant* **43**: 447–454.

Maggini J, Mirkin G, Bognanni I, Holmberg J, Piazzón IM, Nepomnaschy I, Costa H, Cañones C, Raiden S, Vermeulen M, et al. 2010. Mouse bone marrow-derived mesenchymal stromal cells turn activated macrophages into a regulatory-like profile. *PLoS One* **5**.

Maijenburg MW, Kleijer M, Vermeul K, Mul EPJ, van Alphen FPJ, van der Schoot CE, Voermans C. 2012. The composition of the mesenchymal stromal cell compartment in human bone marrow changes during development and aging. *Haematologica* **97**: 179–183.

Marklein RA, Lo Surdo JL, Bellayr IH, Godil SA, Puri RK, Bauer SR. 2016. High Content Imaging of Early Morphological Signatures Predicts Long Term Mineralization Capacity of Human Mesenchymal Stem Cells upon Osteogenic Induction. *Stem Cells* **34**: 935–947.

Marquez-Curtis LA, Janowska-Wieczorek A, McGann LE, Elliott JAW. 2015. Mesenchymal stromal cells derived from various tissues: Biological, clinical and cryopreservation aspects. *Cryobiology* **71**: 181–197.

Martin-Rendon E, Hale SJM, Ryan D, Baban D, Forde SP, Roubelakis M, Sweeney D, Moukayed M, Harris AL, Davies K, et al. 2007. Transcriptional Profiling of Human Cord Blood CD133 + and Cultured Bone Marrow Mesenchymal Stem Cells in Response to Hypoxia . *Stem Cells* **25**: 1003–1012.

Martin PJ, Uberti J, Soiffer R, Klingemann H, Waller EK, Daly A, Herrmann RP, Kebriaei P. 2010. Prochymal Improves Response Rates In Patients With Steroid-Refractory Acute Graft Versus Host Disease (SR-GVHD) Involving The Liver And Gut: Results Of A Randomized, Placebo-Controlled, Multicenter Phase III Trial In GVHD. *Biol Blood Marrow Transplant*.

Mattar P, Bieback K. 2015. Comparing the immunomodulatory properties of bone marrow, adipose tissue, and birth-associated tissue mesenchymal stromal cells. *Front Immunol* **6**.

Mauney JR, Kaplan DL, Volloch V. 2004. Matrix-mediated retention of osteogenic differentiation potential by human adult bone marrow stromal cells during ex vivo expansion. *Biomaterials* **25**: 3233–3243.

Mazur P. 1970. Cryobiology: The freezing of biological systems. *Science (80-)* **168**: 939–49.

McGann LE. 1978. Differing actions of penetrating and nonpenetrating cryoprotective agents. *Cryobiology* **15**: 382–390.

McGann LE, Yang H, Walterson M. 1988. Manifestations of cell damage after freezing and thawing. *Cryobiology* **25**: 178–185.

Meisel R, Zibert A, Laryea M, Göbel U, Däubener W, Dilloo D. 2004. Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated

tryptophan degradation. *Blood* **103**: 4619–21.
Melief SM, Schrama E, Brugman MH, Tiemessen MM, Hoogduijn MJ, Fibbe WE, Roelofs H. 2013. Multipotent stromal cells induce human regulatory T cells through a novel pathway involving skewing of monocytes toward anti-inflammatory macrophages. *Stem Cells* **31**: 1980–1991.
Menard C, Pacelli L, Bassi G, Dulong J, Bifari F, Bezier I, Zanoncello J, Ricciardi M, Latour M, Bourin P, et al. 2013. Clinical-grade mesenchymal stromal cells produced under various good manufacturing practice processes differ in their immunomodulatory properties: Standardization of immune quality controls. *Stem Cells Dev* **22**: 1789–1801.
Mencattini A, Di Giuseppe D, Comes MC, Casti P, Corsi F, Bertani FR, Ghibelli L, Businaro L, Di Natale C, Parrini MC, et al. 2020. Discovering the hidden messages within cell trajectories using a deep learning approach for in vitro evaluation of cancer drug treatments. *Sci Rep* **10**: 7653.
Mendicino M, Bailey AM, Wonnacott K, Puri RK, Bauer SR. 2014. MSC-based product characterization for clinical trials: An FDA perspective. *Cell Stem Cell* **14**: 141–145.
Mesimäki K, Lindroos B, Törnwall J, Mauno J, Lindqvist C, Kontio R, Miettinen S, Suuronen R. 2009. Novel maxillary reconstruction with ectopic bone formation by GMP adipose stem cells. *Int J Oral Maxillofac Surg* **38**: 201–209.
Mets T, Verdonk G. 1981. In vitro aging of human bone marrow derived stromal cells. *Mech Ageing Dev*.
Michler RE. 2018. The current status of stem cell therapy in ischemic heart disease. *J Card Surg* **33**: 520–531.
Minieri V, Saviozzi S, Gambarotta G, Lo Iacono M, Accomasso L, Cibrario Rocchietti E, Gallina C, Turinetto V, Giachino C. 2015. Persistent DNA damage-induced premature senescence alters the functional features of human bone marrow mesenchymal stem cells. *J Cell Mol Med* **19**: 734–743.
Mizukami A, Swiech K. 2018. Mesenchymal stromal cells: From discovery to manufacturing and commercialization. *Stem Cells Int* **2018**.
Mo M, Wang S, Zhou Y, Li H, Wu Y. 2016. Mesenchymal stem cell subpopulations: phenotype, property and therapeutic potential. *Cell Mol Life Sci* **73**: 3311–3321.
Moll G, Alm JJ, Davies LC, Von Bahr L, Heldring N, Stenbeck-Funke L, Hamad OA, Hinsch R, Ignatowicz L, Locke M, et al. 2014a. Do cryopreserved mesenchymal stromal cells display impaired immunomodulatory and therapeutic properties? *Stem Cells* **32**: 2430–2442.
Moll G, Ankrum JA, Kamhieh-Milz J, Bieback K, Ringdén O, Volk HD, Geissler S, Reinke P. 2019. Intravascular Mesenchymal Stromal/Stem Cell Therapy Product Diversification: Time for New Clinical Guidelines. *Trends Mol Med* **25**: 149–163.
Moll G, Drzeniek N, Kamhieh-Milz J, Geissler S, Volk HD, Reinke P. 2020a. MSC Therapies for COVID-19: Importance of Patient Coagulopathy, Thromboprophylaxis, Cell Product Quality and Mode of Delivery for Treatment Safety and Efficacy. *Front Immunol* **11**: 1–10.
Moll G, Geißler S, Catar R, Ignatowicz L, Hoogduijn MJ, Strunk D, Bieback K, Ringdén O. 2016. Cryopreserved or fresh mesenchymal stromal cells: Only a matter of taste or key to unleash the full clinical potential of MSC therapy? *Adv Exp Med Biol* **951**: 77–98.
Moll G, Hoogduijn MJ, Ankrum JA. 2020b. Editorial: Safety, Efficacy and Mechanisms of Action of Mesenchymal Stem Cell Therapies. *Front Immunol* **11**: 1–4.
Moll G, Hult A, Von Bahr L, Alm JJ, Heldring N, Hamad OA, Stenbeck-Funke L, Larsson S, Teramura Y, Roelofs H, et al. 2014b. Do ABO blood group antigens hamper the therapeutic efficacy of mesenchymal stromal cells? *PLoS One* **9**.
Moll G, Jitschin R, Bahr Lena V, Rasmusson-Duprez I, Sundberg B, Lönnies L, Elgue G, Nilsson-Ekdahl K, Mougiakakos D, Lambris JD, et al. 2011. Mesenchymal stromal cells engage complement and complement receptor bearing innate effector cells to modulate immune responses. *PLoS One* **6**: e21703.
Moll G, Rasmusson-Duprez I, Von Bahr L, Connolly-Andersen AM, Elgue G, Funke L, Hamad OA, Lönnies H, Magnusson PU, Sanchez J, et al. 2012. Are therapeutic human mesenchymal stromal cells compatible with human blood? *Stem Cells*.
Moretta L, Bottino C, Cantoni C, Mingari MC, Moretta A. 2001. Human natural killer cell function and receptors. *Curr Opin Pharmacol*.

Morris TJ, Picken A, Sharp DMC, Slater NKH, Hewitt CJ, Coopman K. 2016. The effect of Me2SO overexposure during cryopreservation on HOS TE85 and hMSC viability, growth and quality. *Cryobiology* **73**: 367–375.

Moscoso I, Centeno A, López E, Rodriguez-Barbosa JI, Santamarina I, Filgueira P, Sánchez MJ, Domínguez-Perles R, Peñuelas-Rivas G, Domenech N. 2005. Differentiation "in vitro" of primary and immortalized porcine mesenchymal stem cells into cardiomyocytes for cell transplantation. *Transplant Proc* **37**: 481–2.

Moyzis RK, Buckingham JM, Cram LS, Dani M, Deaven LL, Jones MD, Meyne J, Ratliff RL, Wu JR. 1988. A highly conserved repetitive DNA sequence, (TTAGGG)(n), present at the telomeres of human chromosomes. *Proc Natl Acad Sci U S A* **85**: 6622–6626.

Muguruma Y, Yahata T, Miyatake H, Sato T, Uno T, Itoh J, Kato S, Ito M, Hotta T, Ando K. 2006. Reconstitution of the functional human hematopoietic microenvironment derived from human mesenchymal stem cells in the murine bone marrow compartment. *Blood*.

Müller I, Kordowich S, Holzwarth C, Spano C, Isensee G, Staiber A, Viebahn S, Gieseke F, Langer H, Gawaz MP, et al. 2006. Animal serum-free culture conditions for isolation and expansion of multipotent mesenchymal stromal cells from human BM. *Cytotherapy*.

Naji A, Eitoku M, Favier B, Deschaseaux F, Rouas-Freiss N, Suganuma N. 2019. Biological functions of mesenchymal stem cells and clinical implications. *Cell Mol Life Sci* **76**: 3323–3348.

National Institute of Health (NIH). ImageJ - Image Processing and Analysis in Java. https://imagej.nih.gov/ij/.

Negi N, Griffin MD. 2020. Effects of mesenchymal stromal cells on regulatory T cells: Current understanding and clinical relevance. *Stem Cells* **38**: 596–605.

Németh K, Leelahavanichkul A, Yuen PST, Mayer B, Parmelee A, Doi K, Robey PG, Leelahavanichkul K, Koller BH, Brown JM, et al. 2009. Bone marrow stromal cells attenuate sepsis via prostaglandin E 2-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat Med* **15**: 42–9.

Nishio K, Inoue A. 2005. Senescence-associated alterations of cytoskeleton: Extraordinary production of vimentin that anchors cytoplasmic p53 in senescent human fibroblasts. *Histochem Cell Biol* **123**: 263–273.

Noh H Bin, Ahn HJ, Lee WJ, Kwack KB, Kwon Y Do. 2010. The molecular signature of in vitro senescence in human mesenchymal stem cells. *Genes and Genomics* **32**: 87–93.

Nold P, Hackstein H, Riedlinger T, Kasper C, Neumann A, Mernberger M, Fölsch C, Schmitt J, Fuchs-Winkelmann S, Barckhausen C, et al. 2015. Immunosuppressive capabilities of mesenchymal stromal cells are maintained under hypoxic growth conditions and after gamma irradiation. *Cytotherapy* **17**: 152–162.

Oikawa S, Kawanishi S. 1999. Site-specific DNA damage at GGG sequence by oxidative stress may accelerate telomere shortening. *FEBS Lett* **453**: 365.

Oja S, Kaartinen T, Ahti M, Korhonen M, Laitinen A, Nystedt J. 2019. The utilization of freezing steps in mesenchymal stromal cell (MSC) manufacturing: Potential impact on quality and cell functionality attributes. *Front Immunol* **10**.

Oja S, Komulainen P, Penttilä A, Nystedt J, Korhonen M. 2018. Automated image analysis detects aging in clinical-grade mesenchymal stromal cell cultures. *Stem Cell Res Ther* **9**.

Orozco L, Munar A, Soler R, Alberca M, Soler F, Huguet M, Sentís J, Sánchez A, García-Sancho J. 2013. Treatment of knee osteoarthritis with autologous mesenchymal stem cells: A pilot study. *Transplantation* **95**: 1535–1541.

Oryan A, Kamali A, Moshirib A, Eslaminejad MB. 2017. Role of Mesenchymal Stem Cells in Bone Regenerative Medicine: What Is the Evidence? *Cells Tissues Organs* **204**: 59–83.

Oswald J, Boxberger S, Jorgensen B, Feldmann S, Ehninger G, Bornhäuser M, Werner C. 2004. Mesenchymal Stem Cells Can Be Differentiated Into Endothelial Cells In Vitro. *Stem Cells* **22**: 377–384.

Panés J, García-Olmo D, Van Assche G, Colombel JF, Reinisch W, Baumgart DC, Dignass A, Nachury M, Ferrante M, Kazemi-Shirazi L, et al. 2016. Expanded allogeneic adipose-derived mesenchymal stem cells (Cx601) for complex perianal fistulas in Crohn's disease: a phase 3 randomised, double-blind controlled trial. *Lancet* **388**: 1281–1290.

Panés J, García-Olmo D, Van Assche G, Colombel JF, Reinisch W, Baumgart DC, Dignass A,

Nachury M, Ferrante M, Kazemi-Shirazi L, et al. 2018. Long-term Efficacy and Safety of Stem Cell Therapy (Cx601) for Complex Perianal Fistulas in Patients With Crohn's Disease. *Gastroenterology* **154**: 1334-1342.e4.

Papait A, Vertua E, Magatti M, Ceccariglia S, De Munari S, Silini AR, Sheleg M, Ofir R, Parolini O. 2020. Mesenchymal Stromal Cells from Fetal and Maternal Placenta Possess Key Similarities and Differences: Potential Implications for Their Applications in Regenerative Medicine. *Cells* **9**: 127.

Parolini O, Alviano F, Bagnara GP, Bilic G, Bühring H-J, Evangelista M, Hennerbichler S, Liu B, Magatti M, Mao N, et al. 2008. Concise Review: Isolation and Characterization of Cells from Human Term Placenta: Outcome of the First International Workshop on Placenta Derived Stem Cells. *Stem Cells* **26**: 300–311.

Passos JF, Saretzki G, von Zglinicki T. 2007. DNA damage in telomeres and mitochondria during cellular senescence: Is there a connection? *Nucleic Acids Res* **35**: 7505–7513.

Patki S, Kadam S, Chandra V, Bhonde R. 2010. Human breast milk is a rich source of multipotent mesenchymal stem cells. *Hum Cell* **23**: 35–40.

Peffers MJ, Collins J, Fang Y, Goljanek-Whysall K, Rushton M, Loughlin J, Proctor C, Clegg PD. 2016. Age-related changes in mesenchymal stem cells identified using a multi-omics approach. *Eur Cells Mater* **31**: 136–159.

Perico N, Casiraghi F, Introna M, Gotti E, Todeschini M, Cavinato RA, Capelli C, Rambaldi A, Cassis P, Rizzo P, et al. 2011. Autologous mesenchymal stromal cells and kidney transplantation: A pilot study of safety and clinical feasibility. *Clin J Am Soc Nephrol* **6**: 412–422.

Petersen S, Saretzki G, Von Zglinicki T. 1998. Preferential accumulation of single-stranded regions in telomeres of human fibroblasts. *Exp Cell Res* **239**: 152–160.

Petite H, Viateau V, Bensaïd W, Meunier A, De Pollak C, Bourguignon M, Oudina K, Sedel L, Guillemin G. 2000. Tissue-engineered bone regeneration. *Nat Biotechnol* **18**: 959–963.

Phinney DG, Prockop DJ. 2007. Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation …. *Stem Cells* **25**: 2896–2902.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. 1999a. Multilineage potential of adult human mesenchymal stem cells. *Science (80-)* **284**: 143–147.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. 1999b. Multilineage potential of adult human mesenchymal stem cells. *Science (80-)* **284**: 143–147.

Polchert D, Sobinsky J, Douglas GW, Kidd M, Moadsiri A, Reina E, Genrich K, Mehrotra S, Setty S, Smith B, et al. 2008. IFN-γ activation of mesenchymal stem cells for treatment and prevention of graft versus host disease. *Eur J Immunol* **38**: 1745–55.

Pollock K, Samsonraj RM, Dudakovic A, Thaler R, Stumbras A, McKenna DH, Dosa PI, Van Wijnen AJ, Hubel A. 2017. Improved Post-Thaw Function and Epigenetic Changes in Mesenchymal Stromal Cells Cryopreserved Using Multicomponent Osmolyte Solutions. *Stem Cells Dev* **26**: 828–842.

Pollock K, Sumstad D, Kadidlo D, McKenna DH, Hubel A. 2015. Clinical mesenchymal stromal cell products undergo functional changes in response to freezing. *Cytotherapy* **17**: 38–45.

Prasad VK, Lucas KG, Kleiner GI, Talano JAM, Jacobsohn D, Broadwater G, Monroy R, Kurtzberg J. 2011. Efficacy and Safety of Ex Vivo Cultured Adult Human Mesenchymal Stem Cells (Prochymal™) in Pediatric Patients with Severe Refractory Acute Graft-Versus-Host Disease in a Compassionate Use Study. *Biol Blood Marrow Transplant* **17**: 534–541.

Prasanna SJ, Gopalakrishnan D, Shankar SR, Vasandan AB. 2010. Pro-inflammatory cytokines, IFNγ and TNFα, influence immune properties of human bone marrow and Wharton jelly mesenchymal stem cells differentially. *PLoS One* **5**.

Prockop DJ, Sekiya I, Colter DC. 2001. Isolation and characterization of rapidly self-renewing stem cells from cultures of human marrow stromal cells. *Cytotherapy* **3**: 393–396.

Prowse KR, Greider CW. 1995. Developmental and tissue-specific regulation of mouse telomerase and telomere length. *Proc Natl Acad Sci U S A* **92**: 4818–4822.

Puck TT, Cieciura SJ, Robinson A. 1958. Genetics of somatic mammalian cells. III. Long-term cultivation of euploid cells from human and animal subjects. *J Exp Med*.

Purpura KA. 2004. Sustained In Vitro Expansion of Bone Progenitors Is Cell Density Dependent. *Stem Cells* **22**: 39-50.
Quinn C, Flake AW. 2008. In vivo differentiation potential of mesenchymal stem cells: Prenatal and postnatal model systems. *Transfus Med Hemotherapy* **35**: 239–247.
Rasmusson I, Le Blanc K, Sundberg B, Ringdén O. 2007. Mesenchymal stem cells stimulate antibody secretion in human B cells. *Scand J Immunol* **65**: 336–343.
Redondo-Castro E, Cunningham C, Miller J, Martuscelli L, Aoulad-Ali S, Rothwell NJ, Kielty CM, Allan SM, Pinteaux E. 2017. Interleukin-1 primes human mesenchymal stem cells towards an anti-inflammatory and pro-trophic phenotype in vitro. *Stem Cell Res Ther* **8**: 1–11.
Reinders MEJ, Dreyer GJ, Bank JR, Roelofs H, Heidt S, Roelen DL, Zandvliet ML, Huurman VAL, Fibbe WE, Kooten C, et al. 2015. Safety of allogeneic bone marrow derived mesenchymal stromal cell therapy in renal transplant recipients: The neptune study. *J Transl Med* **13**.
Reinisch A, Etchart N, Thomas D, Hofmann NA, Fruehwirth M, Sinha S, Chan CK, Senarath-Yapa K, Seo EY, Wearda T, et al. 2015. Epigenetic and in vivo comparison of diverse MSC sources reveals an endochondral signature for human hematopoietic niche formation. *Blood* **125**: 249–260.
Ribeiro A, Laranjeira P, Mendes S, Velada I, Leite C, Andrade P, Santos F, Henriques A, Grãos M, Cardoso CMP, et al. 2013. Mesenchymal stem cells from umbilical cord matrix, adipose tissue and bone marrow exhibit different capability to suppress peripheral blood B, natural killer and T cells. *Stem Cell Res Ther* **4**.
Ringdén O, Uzunel M, Rasmusson I, Remberger M, Sundberg B, Lönnies H, Marschall HU, Dlugosz A, Szakos A, Hassan Z, et al. 2006. Mesenchymal stem cells for treatment of therapy-resistant graft-versus-host disease. *Transplantation* **81**: 1390–1397.
Ringe J, Häupl T, Sittinger M. 2003. Mesenchymal Stem Cells for Tissue Engineering of Bone and Cartilage. *Med Klin*.
Ross SE, Hemati N, Longo KA, Bennett CN, Lucas PC, Erickson RL, MacDougald OA. 2000. Inhibition of adipogenesis by Wnt signaling. *Science (80-)* **289**: 950–953.
Russell AL, Lefavor RC, Zubair AC. 2018. Characterization and cost–benefit analysis of automated bioreactor-expanded mesenchymal stem cells for clinical applications. *Transfusion* **58**: 2374–2382.
Sacchetti B, Funari A, Michienzi S, Di Cesare S, Piersanti S, Saggio I, Tagliafico E, Ferrari S, Robey PG, Riminucci M, et al. 2007. Self-Renewing Osteoprogenitors in Bone Marrow Sinusoids Can Organize a Hematopoietic Microenvironment. *Cell* **131**: 324–336.
Sacchetti B, Funari A, Remoli C, Giannicola G, Kogler G, Liedtke S, Cossu G, Serafini M, Sampaolesi M, Tagliafico E, et al. 2016. No identical "mesenchymal stem cells" at different times and sites: Human committed progenitors of distinct origin and differentiation potential are incorporated as adventitial cells in microvessels. *Stem Cell Reports* **6**: 897–913.
Salmenniemi U, Itälä-Remes M, Nystedt J, Putkonen M, Niittyvuopio R, Vettenranta K, Korhonen M. 2017. Good responses but high TRM in adult patients after MSC therapy for GvHD. *Bone Marrow Transplant* **52**: 606–608.
Samuelsson H, Ringdén O, Lonnies H, Le Blanc K. 2009. Optimizing in vitro conditions for immunomodulation and expansion of mesenchymal stromal cells. *Cytotherapy* **11**: 129–36.
Santa María L, Rojas C V., Minguell JJ. 2004. Signals from damaged but not undamaged skeletal muscle induce myogenic differentiation of rat bone-marrow-derived mesenchymal stem cells. *Exp Cell Res*.
Saretzki G, von Zglinicki T. 1999. Replicative senescence as a model for aging: The role of oxidative stress and telomere shortening. *Z Gerontol Geriatr*.
Sarugaser R, Lickorish D, Baksh D, Hosseini MM, Davies JE. 2005. Human Umbilical Cord Perivascular (HUCPV) Cells: A Source of Mesenchymal Progenitors. *Stem Cells* **23**: 220–229.
Sasaki H, Takeuchi I, Okada M, Sawada R, Kanie K, Kiyota Y, Honda H, Kato R. 2014. Label-free morphology-based prediction of multiple differentiation potentials of human

mesenchymal stem cells for early evaluation of intact cells. *PLoS One* **9**.
Schallmoser K, Bartmann C, Rohde E, Reinisch A, Kashofer K, Stadelmeyer E, Drexler C, Lanzer G, Linkesch W, Strunk D. 2007. Human platelet lysate can replace fetal bovine serum for clinical-scale expansion of functional mesenchymal stromal cells. *Transfusion* **47**: 1436–1446.
Schellenberg A, Hemeda H, Wagner W. 2013. Tracking of replicative senescence in mesenchymal stem cells by colony-forming unit frequency. *Methods Mol Biol*.
Schellenberg A, Mauen S, Koch CM, Jans R, De Waele P, Wagner W. 2014. Proof of principle: Quality control of therapeutic cell preparations using senescence-associated DNA-methylation changes. *BMC Res Notes*.
Schieker M, Gülkan H, Austrup B, Neth P, Mutschler W. 2004. Telomerase activity and telomere length of human mesenchymal stem cells. Changes during osteogenic differentiation. *Orthopade* **33**: 1373–1377.
Schneider CK, Celis P, Salmikangas P, Figuerola-Santos MA, D'Apote L, Oliver-Diaz O, Büttel I, Mačiulaitis R, Robert JL, Silva Lima B, et al. 2010. Challenges with advanced therapy medicinal products and how to meet them. *Nat Rev Drug Discov* **9**: 195–201.
Scolding NJ, Pasquini M, Reingold SC, Cohen JA. 2017. Cell-based therapeutic strategies for multiple sclerosis. *Brain* **140**: 2776–2796.
Sekiya I, Larson BL, Smith J, Pochampally R, Cui J-G, Prockop DJ. 2002. Expansion of Human Adult Stem Cells from Bone Marrow Stroma: Conditions that Maximize the Yields of Early Progenitors and Evaluate Their Quality. *Stem Cells*.
Selmani Z, Naji A, Zidi I, Favier B, Gaiffe E, Obert L, Borg C, Saas P, Tiberghien P, Rouas-Freiss N, et al. 2008. Human Leukocyte Antigen-G5 Secretion by Human Mesenchymal Stem Cells Is Required to Suppress T Lymphocyte and Natural Killer Function and to Induce CD4 + CD25 high FOXP3 + Regulatory T Cells. *Stem Cells* **26**: 212–222.
Selvaggi TA, Walker RE, Fleisher TA. 1997. Development of antibodies to fetal calf serum with arthus-like reactions in human immunodeficiency virus-infected patients given syngeneic lymphocyte infusions. *Blood*.
Sensebé L. 2008. Clinical grade production of mesenchymal stem cells. *Biomed Mater Eng*.
Sensebé L, Gadelorge M, Fleury-Cappellesso S. 2013. Production of mesenchymal stromal/stem cells according to good manufacturing practices: A review. *Stem Cell Res Ther* **4**.
Sepúlveda CJ, Tomé M, Eugenia Fernández M, Delgado M, Campisi J, Bernad A, González MA. 2014. Cell senescence abrogates the therapeutic potential of human mesenchymal stem cells in the lethal endotoxemia model. *Stem Cells* **32**: 1865–1877.
Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. 1997. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16(INK4a). *Cell* **88**: 593–602.
Sethe S, Scutt A, Stolzing A. 2006. Aging of mesenchymal stem cells. *Ageing Res Rev* **5**: 91–116.
Severino J, Allen RG, Balin S, Balin A, Cristofalo VJ. 2000. Is β-galactosidase staining a marker of senescence in vitro and in vivo? *Exp Cell Res* **257**: 162–171.
Shahdadfar A, Frønsdal K, Haug T, Reinholt FP, Brinchmann JE. 2005. In Vitro Expansion of Human Mesenchymal Stem Cells: Choice of Serum Is a Determinant of Cell Proliferation, Differentiation, Gene Expression, and Transcriptome Stability. *Stem Cells* **23**: 1357–1366.
Sherr CJ, DePinho RA. 2000. Mitotic Clock or Culture Shock? *Cell* **102**: 407–410.
Sherr CJ, Roberts JM. 1999. CDK inhibitors: Positive and negative regulators of G1-phase progression. *Genes Dev* **13**: 1501–12.
Shih DT, Lee D-C, Chen S-C, Tsai R-Y, Huang C-T, Tsai C-C, Shen E-Y, Chiu W-T. 2005. Isolation and Characterization of Neurogenic Mesenchymal Stem Cells in Human Scalp Tissue. *Stem Cells*.
Shim WSN, Jiang S, Wong P, Tan J, Chua YL, Seng Tan Y, Sin YK, Lim CH, Chua T, Teh M, et al. 2004. Ex vivo differentiation of human adult bone marrow stem cells into cardiomyocyte-like cells. *Biochem Biophys Res Commun* **324**: 481–488.
Singer NG, Caplan AI. 2011. Mesenchymal Stem Cells: Mechanisms of Inflammation. *Annu Rev Pathol Mech Dis*.
Sipp D, Robey PG, Turner L. 2018. Clear up this stem cell mess. *Nature* **561**: 455–457.
Skolekova S, Matuskova M, Bohac M, Toro L, Demkova L, Gursky J, Kucerova L. 2016. Cisplatin-

induced mesenchymal stromal cells-mediated mechanism contributing to decreased antitumor effect in breast cancer cells. *Cell Commun Signal* **14**.
Sogo Y, Ito A, Matsuno T, Oyane A, Tamazawa G, Satoh T, Yamazaki A, Uchimura E, Ohno T. 2007. Fibronectin-calcium phosphate composite layer on hydroxyapatite to enhance adhesion, cell spread and osteogenic differentiation of human mesenchymal stem cells in vitro. *Biomed Mater* **2**: 116–123.
Soler R, Orozco L, Munar A, Huguet M, López R, Vives J, Coll R, Codinach M, Garcia-Lopez J. 2016. Final results of a phase I–II trial using ex vivo expanded autologous Mesenchymal Stromal Cells for the treatment of osteoarthritis of the knee confirming safety and suggesting cartilage regeneration. *Knee* **23**: 647–654.
Sotiropoulou PA, Perez SA, Gritzapis AD, Baxevanis CN, Papamichail M. 2006. Interactions Between Human Mesenchymal Stem Cells and Natural Killer Cells. *Stem Cells* **24**: 74–85.
Spaggiari GM, Capobianco A, Becchetti S, Mingari MC, Moretta L. 2006. Mesenchymal stem cell-natural killer cell interactions: Evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation. *Blood* **107**: 1484–1490.
Spees JL, Gregory CA, Singh H, Tucker HA, Peister A, Lynch PJ, Hsu SC, Smith J, Prockop DJ. 2004. Internalized antigens must be removed to prepare hypoimmunogenic mesenchymal stem cells for cell and gene therapy. *Mol Ther* **9**: 747–56.
Stagg J, Pommey S, Eliopoulos N, Galipeau J. 2006. Interferon-γ-stimulated marrow stromal cells: A new type of nonhematopoietic antigen-presenting cell. *Blood* **107**: 2570–2577.
Stanovici J, Le Nail LR, Brennan MA, Vidal L, Trichet V, Rosset P, Layrolle P. 2016. Bone regeneration strategies with bone marrow stromal cells in orthopaedic surgery. *Curr Res Transl Med* **64**: 83–90.
Stein GH, Drullinger LF, Soulard A, Dulic V. 1999. Differential Roles for Cyclin-Dependent Kinase Inhibitors p21 and p16 in the Mechanisms of Senescence and Differentiation in Human Fibroblasts. *Mol Cell Biol* **19**: 2109–2117.
Stenderup K, Justesen J, Clausen C, Kassem M. 2003. Aging is associated with decreased maximal life span and accelerated senescence of bone marrow stromal cells. *Bone* **33**: 919–926.
Stolzing A, Jones E, McGonagle D, Scutt A. 2008. Age-related changes in human bone marrow-derived mesenchymal stem cells: Consequences for cell therapies. *Mech Ageing Dev* **129**: 163–73.
Strandberg G, Sellberg F, Sommar P, Ronaghi M, Lubenow N, Knutson F, Berglund D. 2017. Standardizing the freeze-thaw preparation of growth factors from platelet lysate. *Transfusion* **57**: 1058–1065.
Strioga M, Viswanathan S, Darinskas A, Slaby O, Michalek J. 2012. Same or not the same? comparison of adipose tissue-derived versus bone marrow-derived mesenchymal stem and stromal cells. *Stem Cells Dev* **21**: 2724–2752.
Tarte K, Gaillard J, Lataillade J-J, Fouillard L, Becker M, Mossafa H, Tchirkov A, Ne Rouard H, Henry C, Splingard M, et al. 2010. Clinical-grade production of human mesenchymal stromal cells: occurrence of aneuploidy without transformation. *Blood* **115**: 3541–3552. www.bloodjournal.org.
Timper K, Seboek D, Eberhardt M, Linscheid P, Christ-Crain M, Keller U, Müller B, Zulewski H. 2006. Human adipose tissue-derived mesenchymal stem cells differentiate into insulin, somatostatin, and glucagon expressing cells. *Biochem Biophys Res Commun* **341**: 1135–1140.
Tolar J, Le Blanc K, Keating A, Blazar BR. 2010. Concise review: Hitting the right spot with mesenchymal stromal cells. *Stem Cells* **28**: 1446–1455.
Tormin A, Li O, Brune JC, Walsh S, Schütz B, Ehinger M, Ditzel N, Kassem M, Scheding S. 2011. CD146 expression on primary nonhematopoietic bone marrow stem cells is correlated with in situ localization. *Blood* **117**: 5067–5077.
Toussaint O, Medrano EE, von Zglinicki T. 2000. Cellular and molecular mechanisms of stress-induced premature senescence (SIPS) of human diploid fibroblasts and melanocytes. *Exp Gerontol* **35**: 927–45.
Traggiai E, Volpi S, Schena F, Gattorno M, Ferlito F, Moretta L, Martini A. 2008. Bone Marrow-

Derived Mesenchymal Stem Cells Induce Both Polyclonal Expansion and Differentiation of B Cells Isolated from Healthy Donors and Systemic Lupus Erythematosus Patients. *Stem Cells* **26**: 562–569.
Troyer D, Weiss ML. 2008. Wharton's Jelly-Derived Cells Are a Primitive Stromal Cell Population. *Stem Cells* **26**: 591–599.
Tsai CC, Chen YJ, Yew TL, Chen LL, Wang JY, Chiu CH, Hung SC. 2011. Hypoxia inhibits senescence and maintains mesenchymal stem cell properties through down-regulation of E2A-p21 by HIF-TWIST. *Blood* **117**: 459–469.
Turinetto V, Vitale E, Giachino C. 2016. Senescence in human mesenchymal stem cells: Functional changes and implications in stem cell-based therapy. *Int J Mol Sci* **17**.
Tyndall A, Uccelli A. 2009. Multipotent mesenchymal stromal cells for autoimmune diseases: Teaching new dogs old tricks. *Bone Marrow Transplant* **43**: 821–828.
Uccelli A, Moretta L, Pistoia V. 2008. Mesenchymal stem cells in health and disease. *Nat Rev Immunol* **8**: 726–736.
Van der Valk J, Bieback K, Buta C, Cochrane B, Dirks WG, Fu J, Hickman JJ, Hohensee C, Kolar R, Liebsch M, et al. 2018. Fetal Bovine Serum (FBS): Past - Present - Future. *ALTEX* **35**: 99–118.
van der Valk J, Brunner D, De Smet K, Fex Svenningsen Å, Honegger P, Knudsen LE, Lindl T, Noraberg J, Price A, Scarino ML, et al. 2010. Optimization of chemically defined cell culture media - Replacing fetal bovine serum in mammalian in vitro methods. *Toxicol Vitr*.
Vanikar A V., Trivedi HL, Kumar A, Gopal SC, Patel H V., Gumber MR, Kute VB, Shah PR, Dave SD. 2014. Co-infusion of donor adipose tissue-derived mesenchymal and hematopoietic stem cells helps safe minimization of immunosuppression in renal transplantation - Single center experience. *Ren Fail* **36**: 1376–1384.
Vaziri H, Benchimol S. 1998. Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. *Curr Biol* **8**: 279–282.
Viswanathan S, Shi Y, Galipeau J, Krampera M, Leblanc K, Martin I, Nolta J, Phinney DG, Sensebé L. 2019. Mesenchymal stem versus stromal cells: International Society for Cell & Gene Therapy (ISCT®) Mesenchymal Stromal Cell committee position statement on nomenclature. *Cytotherapy* **21**: 1019–1024.
Vogel JP, Szalay K, Geiger F, Kramer M, Richter W, Kasten P. 2006. Platelet-rich plasma improves expansion of human mesenchymal stem cells and retains differentiation capacity and in vivo bone formation in calcium phosphate ceramics. *Platelets* **17**: 462–469.
Volkman R, Offen D. 2017. Concise Review: Mesenchymal Stem Cells in Neurodegenerative Diseases. *Stem Cells* **35**: 1867–1880.
Volz AC, Huber B, Kluger PJ. 2016. Adipose-derived stem cell differentiation as a basic tool for vascularized adipose tissue engineering. *Differentiation* **92**: 52–64.
von Bonin M, Stölzel F, Goedecke A, Richter K, Wuschek N, Hölig K, Platzbecker U, Illmer T, Schaich M, Schetelig J, et al. 2009. Treatment of refractory acute GVHD with third-party MSC expanded in platelet lysate-containing medium. *Bone Marrow Transplant* **43**: 245–251.
von Zglinicki T. 2002. Oxidative stress shortens telomeres. *Trends Biochem Sci* **27**: 339–44.
von Zglinicki T, Pilger R, Sitte N. 2000. Accumulation of single-strand breaks is the major cause of telomere shortening in human fibroblasts. *Free Radic Biol Med* **28**: 64–74.
von Zglinicki T, Saretzki G, Döcke W, Lotze C. 1995. Mild Hyperoxia Shortens Telomeres and Inhibits Proliferation of Fibroblasts: A Model for Senescence? *Exp Cell Res* **220**: 186–193.
Wagner W, Bork S, Lepperdinger G, Joussen S, Ma N, Strunk D, Koch CM. 2010. How to track cellular aging of mesenchymal stromal cells? *Aging (Albany NY)* **2**: 224–230.
Wagner W, Frobel J, Goetzke R. 2016. Epigenetic quality check - How good are your mesenchymal stromal cells? *Epigenomics* **8**: 889–94.
Wagner W, Horn P, Castoldi M, Diehlmann A, Bork S, Saffrich R, Benes V, Blake J, Pfister S, Eckstein V, et al. 2008. Replicative senescence of mesenchymal stem cells: A continuous and organized process. *PLoS One* **3**: e2213.
Wagner W, Wein F, Seckinger A, Frankhauser M, Wirkner U, Krause U, Blake J, Schwager C, Eckstein V, Ansorge W, et al. 2005. Comparative characteristics of mesenchymal stem cells from human bone marrow, adipose tissue, and umbilical cord blood. *Exp Hematol* **33**:

1402–1416.
Wang D, Li J, Zhang Y, Zhang M, Chen J, Li X, Hu X, Jiang S, Shi S, Sun L. 2014. Umbilical cord mesenchymal stem cell transplantation in active and refractory systemic lupus erythematosus: A multicenter clinical study. *Arthritis Res Ther* **16**.
Warnke PH, Humpe A, Strunk D, Stephens S, Warnke F, Wiltfang J, Schallmoser K, Alamein M, Bourke R, Heiner P, et al. 2013. A clinically-feasible protocol for using human platelet lysate and mesenchymal stem cells in regenerative therapies. *J Cranio-Maxillofacial Surg* **41**: 153–161.
Wei W, Sedivy JM. 1999. Differentiation between senescence (M1) and crisis (M2) in human fibroblast cultures. *Exp Cell Res*.
Weiss DJ, English K, Krasnodembskaya A, Isaza-Correa JM, Hawthorne IJ, Mahon BP. 2019. The necrobiology of mesenchymal stromal cells affects therapeutic efficacy. *Front Immunol* **10**: 1–12.
Wessman SJ, Levings RL. 1999. Benefits and risks due to animal serum used in cell culture production. *Dev Biol Stand*.
Woodbury D, Schwarz EJ, Prockop DJ, Black IB. 2000. Adult rat and human bone marrow stromal cells differentiate into neurons. *J Neurosci Res* **61**: 364–370.
Wright WE, Piatyszek MA, Rainey WE, Byrd W, Shay JW. 1996. Telomerase activity in human germline and embryonic tissues and cells. *Dev Genet* **18**: 173–179.
Wu LW, Wang YL, Christensen JM, Khalifian S, Schneeberger S, Raimondi G, Cooney DS, Lee WPA, Brandacher G. 2014. Donor age negatively affects the immunoregulatory properties of both adipose and bone marrow derived mesenchymal stem cells. *Transpl Immunol* **30**: 122–127.
Xia W, Li H, Wang Z, Xu R, Fu Y, Zhang X, Ye X, Huang Y, Xiang AP, Yu W. 2011. Human platelet lysate supports ex vivo expansion and enhances osteogenic differentiation of human bone marrow-derived mesenchymal stem cells. *Cell Biol Int* **35**: 639–643.
Yamada Y, Ueda M, Naiki T, Takahashi M, Hata KI, Nagasaka T. 2004. Autogenous injectable bone for regeneration with mesenchymal stem cells and platelet-rich plasma: Tissue-engineered bone regeneration. *Tissue Eng* **10**: 955–964.
Yamout B, Hourani R, Salti H, Barada W, El-Hajj T, Al-Kutoubi A, Herlopian A, Baz EK, Mahfouz R, Khalil-Hamdan R, et al. 2010. Bone marrow mesenchymal stem cell transplantation in patients with multiple sclerosis: A pilot study. *J Neuroimmunol* **227**: 185–189.
Yang H. 2011. South Korea's stem cell approval. *Nat Biotechnol*.
Yang NC, Hu ML. 2005. The limitations and validities of senescence associated-β- galactosidase activity as an aging marker for human foreskin fibroblast Hs68 cells. *Exp Gerontol* **40**: 813–819.
Yong KW, Safwani WKZW, Xu F, Zhang X, Choi JR, Abas WABW, Omar SZ, Azmi MAN, Chua KH, Pingguan-Murphy B. 2017. Assessment of tumourigenic potential in long-term cryopreserved human adipose-derived stem cells. *J Tissue Eng Regen Med* **11**: 2217–2226.
Zajdel A, Kalucka M, Kokoszka-Mikolaj E, Wilczok A. 2017. Osteogenic differentiation of human mesenchymal stem cells from adipose tissue and Wharton's jelly of the umbilical cord. *Acta Biochim Pol* **64**: 365–369.
Zaky SH, Ottonello A, Strada P, Cancedda R, Mastrogiacomo M. 2008. Platelet lysate favours in vitro expansion of human bone marrow stromal cells for bone and cartilage engineering. *J Tissue Eng Regen Med* **2**: 472–481.
Zappia E, Casazza S, Pedemonte E, Benvenuto F, Bonanni I, Gerdoni E, Giunti D, Ceravolo A, Cazzanti F, Frassoni F, et al. 2005. Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy. *Blood* **106**: 1755–1761.
Zhang B, Liu R, Shi D, Liu X, Chen Y, Dou X, Zhu X, Lu C, Liang W, Liao L, et al. 2009. Mesenchymal stem cells induce mature dendritic cells into a novel Jagged-2 dependent regulatory dendritic cell population. *Blood* **113**: 46–57.
Zhang J, Feng F, Wang Q, Zhu X, Fu H, Xu L, Liu K, Huang X, Zhang X. 2016. Platelet-Derived Growth Factor-BB Protects Mesenchymal Stem Cells (MSCs) Derived From Immune Thrombocytopenia Patients Against Apoptosis and Senescence and Maintains MSC-Mediated Immunosuppression. *Stem Cells Transl Med* **5**: 1631–1643.

Zhang XH, Zhang JM, Wang QM, Feng FE, Zhu XL, Lv M, Fu HX, Xu LP, Liu KY, Huang XJ. 2015. PDGF-BB protects bone marrow mesenchymal stem cells against apoptosis and senescence through the P53/P21 pathway in patients with immune thrombocytopaenia. *Haematologica*.

Zhu W, Xu W, Jiang R, Qian H, Chen M, Hu J, Cao W, Han C, Chen Y. 2006. Mesenchymal stem cells derived from bone marrow favor tumor cell growth in vivo. *Exp Mol Pathol* **80**.

Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH. 2002. Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* **13**: 4279–95.